cancer treatment reports

Proceedings of the Symposium on the Metabolism and Mechanism of Action of Cyclophosphamide

Volume 60
Number 4
April 1976
Pages 299-525

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service National Institutes of Health

cancer treatment reports

National Cancer Institute, *Frank J. Rauscher, Jr., Director*

Division of Cancer Treatment, *Vincent T. DeVita, Jr., Director*

Editorial Board

J. A. R. Mead, *Editor-in-Chief*

Associate Editors:

Bruce A. Chabner
David A. Cooney
David G. Johns
Ralph E. Johnson
John S. Macdonald
Stephen C. Schimpff
Susan M. Sieber
John M. Venditti
John L. Ziegler

Editorial Staff

Pamela W. Hutchins, *Managing Editor*
Jean M. Griffin, *Assistant Editor*

Editorial Assistants:

Virginia T. Ascosi
Elaine P. Beck

Advisory Board

Bernard Fisher
Emil J Freireich
Lewis W. Guiss
Simon Kramer
Faith H. Kung
LaSalle D. Leffall, Jr.
John A. Montgomery
David G. Nathan
Paul E. Neiman
Stanley E. Order
Theodore L. Phillips
Philip Rubin
Edward F. Scanlon
Stuart F. Schlossman
Joseph V. Simone

Editorial Policy

The Editorial Board will consider manuscripts dealing with all aspects of preclinical and clinical cancer treatment. These may include such topics as surgery, radiotherapy, chemotherapy, immunotherapy, supportive care, pharmacology, cell biology and kinetics, mechanisms of drug action, and medicinal chemistry.

The journal will consist of two parts. One part will consider previously unpublished manuscripts of original work under four main categories:

1. **Clinical or preclinical research.**
2. **Correspondence regarding manuscripts previously published in *Cancer Treatment Reports*. Letters on other topics will be published at the discretion of the Editorial Board.**
3. **Invited guest editorials and reviews. Unsolicited contributions will be considered on their individual merits.**
4. **Brief communications. Under this category the Editorial Board will consider short contributions which should not exceed five typewritten pages. Four types of communication will be considered in this category.**
   - **(*a*) Ideas relating to cancer treatment.**
   - **(*b*) Preliminary research reports.**
   - **(*c*) Clinical case reports.**
   - **(*d*) Brief clinical trials of novel treatments.**

All material submitted for consideration in the journal will be subject to review when appropriate by at least two outside reviewers and one member of the Editorial Board. The decision to publish a manuscript is solely the responsibility of the Editorial Board.

The second part of this journal will consist of supplements (published bimonthly) which may include proceedings of meetings and symposia and program information from the Division of Cancer Treatment, National Cancer Institute. Requests to publish information in *Cancer Treatment Reports* supplement will be considered by the Editorial Board.

INSTRUCTIONS TO AUTHORS

Summary
Begin the article with a short summary.

Text
Submit an original and three copies of the manuscript, typed double spaced. Each page should begin with a new paragraph. Use accepted spelling, abbreviation, and hyphenation found in *Webster's New International Dictionary*. Footnotes should be typed at the bottom of the page on which they appear.

Tables
Submit each table on a separate page, typed double spaced. Please use standard abbreviations; otherwise spell out.

References
Submit references typed double spaced. Follow the standard form of *Index Medicus:* author, title, name of journal, volume number, page or inclusive pages, and year. In book references give author, title of book, edition (if more than one), city, name of publisher, date of book, volume number, and pages cited. References in text should begin with number 1 and continue consecutively. References to papers presented at meetings should include author; title of paper; title, city, and date of meeting; city and name of publisher; date of publication; volume number; and pages. Papers presented at meetings but not published should be cited in a footnote rather than in the reference section.

Illustrations
Photographs and graphs preferably should be clear, glossy prints. Original art work should be done in black ink for good reproduction. Please send 2 copies of each print.

Nomenclature
Names of compounds should conform to American usage. See *Chemical Abstracts* for proper nomenclature.

Abbreviations
Only standard abbreviations are acceptable. If a new or coined abbreviation is used, it must be defined when first mentioned.

Brief Reports
Manuscripts published in the Brief Reports section can be no longer than 5 double-spaced pages including references and tables. Summaries and figures are usually omitted.

Symposia
Requests to publish the proceedings of symposia should be addressed to the Managing Editor. A copy of the agenda and a list of speakers should accompany such requests.

Submission of Manuscripts
Manuscripts and all inquiries concerning the status of manuscripts should be addressed to: Managing Editor, Cancer Treatment Reports, National Cancer Institute, 8300 Colesville Rd, Rm 3A05, Blair Bldg, Silver Spring Md 20910.

Reprints
Supplied free of charge: 250 reprints will be sent to the senior author about three weeks after publication of the article.

SUBSCRIPTIONS

Cancer Treatment Reports is for sale by the Superintendent of Documents, US Government Printing Office, Washington, DC 20402. The yearly subscription rate is $38.35 domestic, with an additional $9.60 for foreign mailing. The prices vary for individual issues. Payment is required in advance; check or money order should be made payable to the Superintendent of Documents. Please specify List ID "CAN" when requesting a subscription.

Symposium on the Metabolism and Mechanism of Action of Cyclophosphamide

Chester Beatty Research Institute
Institute of Cancer Research

Editors: Peter J. Cox
Peter B. Farmer
Michael Jarman

London, England
July 10-12, 1975

Contents

Cancer Treatment Reports
Vol. 60, No. 4
April 1976

Proceedings of the Symposium on the Metabolism and Mechanism of Action of Cyclophosphamide: July 10-12, 1975, London, England

Page

Introduction 299

Peter J. Cox, Peter B. Farmer, and Michael Jarman
Chester Beatty Research Institute, London, England

Comparative Pharmacologic Study In Vitro and In Vivo With Cyclophosphamide (NSC-26271), Cyclophosphamide Metabolites, and Plain Nitrogen Mustard Compounds 301
N. Brock
Asta-Werke Aktiengesellschaft, Chemische Fabrik, Bielefeld, West Germany

The Problem of Oncostatic Specificity of Cyclophosphamide (NSC-26271): Studies on Reactions that Control the Alkylating and Cytotoxic Activity 309
H.-J. Hohorst, U. Draeger, G. Peter, and G. Voelcker
Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany

Aldophosphamide: Synthesis, Characterization, and Comparison With "Hohorst's Aldophosphamide" 317
Robert F. Struck
Southern Research Institute, Birmingham, Alabama

Studies on the Selective Action of Cyclophosphamide (NSC-26271): Inactivation of the Hydroxylated Metabolite by Tissue-Soluble Enzymes 321
Peter J. Cox, Barry J. Phillips, and Peter Thomas
Institute of Cancer Research, London, England

Studies on the In Vivo Formation of Acrolein: 3-Hydroxypropyl-mercapturic Acid as an Index of Cyclophosphamide (NSC-26271) Activation 327
R. A. Alarcon
Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts

Cyclophosphamide (NSC-26271)-Related Phosphoramide Mustards—Recent Advances and Historical Perspective 337
Orrie M. Friedman, Isidore Wodinsky, and Arthur Myles
Collaborative Research, Inc, Waltham, Massachusetts

Cyclic Phosphoramide Mustard (NSC-69945) Derivatives of Amino Acids and Peptides 347
M. Szekerke
Institute of Organic Chemistry, Eötvös University, Budapest, Hungary

Deactivation of Cyclophosphamide (NSC-26271) Metabolites by Sulfhydryl Compounds 355
U. Draeger, G. Peter, and H.-J. Hohorst
Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany

Stereochemistry, Metabolism, and Antitumor Activity of 4-Hydroperoxyisophosphamide (NSC-227114) and its Stereoisomer 361
A. Takamizawa, T. Iwata, K. Yamaguchi, O. Shiratori, M. Harada, Y. Tochino, and S. Matsumoto
Shionogi and Co, Ltd, Osaka, Japan

Two Stable Fenton Oxidation Products of Cyclophosphamide (NSC-26271) as Precursors of 4-Hydroxycyclophosphamide (NSC-196562) Under Physiologic Conditions 369
Catherine Benckhuysen, Jan van der Steen, and Edmond J. Spanjersberg
The Netherlands Cancer Institute, Amsterdam

Biologic Activity of Two Derivatives and Six Possible Metabolites of Cyclophosphamide (NSC-26271) 373
P. Lelieveld and L. M. van Putten
Radiobiological Institute TNO, Rijswijk, The Netherlands

Synthesis and Structure-Activity Relationships of Pre-activated Analogs of Cyclophosphamide (NSC-26271) 381
John A. Montgomery and Robert F. Struck
Southern Research Institute, Birmingham, Alabama

Importance of Pharmacokinetic Studies on Cyclophosphamide (NSC-26271) in Understanding its Cytotoxic Effect 395
M. G. Donelli, I. Bartošek, A. Guaitani, A. Martini, T. Colombo, M. A. Pacciarini, and R. Modica
Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy

Approaches to the Pharmacokinetics of Cyclophosphamide (NSC-26271): Quantitation of Metabolites 403
Ian Jardine, Robert Brundrett, Michael Colvin, and Catherine Fenselau
The Johns Hopkins University School of Medicine, Baltimore, Maryland

Sparing Effect of Cyclophosphamide (NSC-26271) Pretreatment on Animals Lethally Treated With γ-Irradiation 409
J. L. Millar and B. N. Hudspith
Institute of Cancer Research, Sutton, Surrey, England

Identification and Pharmacokinetics of Cyclophosphamide (NSC-26271) Metabolites In Vivo 415
G. Voelcker, T. Wagner, and H.-J. Hohorst
Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany

Permeation of Cyclophosphamide (NSC-26271) Metabolites Into Tumor Cells 423
U. Draeger and H.-J. Hohorst
Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany

Studies on 4-Hydroperoxycyclophosphamide (NSC-181815): A Simple Preparation Method and its Application for the Synthesis of a New Class of "Activated" Sulfur-Containing Cyclophosphamide (NSC-26271) Derivatives 429
G. Peter, T. Wagner, and H.-J. Hohorst
Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany

Studies on the Metabolism of Isophosphamide (NSC-109724) in Man 437
K. Norpoth
Institüt für Staublungenforschung und Arbeitsmedizin, Universität Münster, West Germany

Studies on the Clinical Pharmacology and Toxicology of Isophosphamide (NSC-109724) 445
Patrick J. Creaven, Larry M. Allen, Martin H. Cohen, and Robert L. Nelson
Roswell Park Memorial Institute, Buffalo, New York

Studies on the Human Pharmacokinetics of Isophosphamide (NSC-109724) 451
Larry M. Allen, Patrick J. Creaven, and Robert L. Nelson
Roswell Park Memorial Institute, Buffalo, New York

Some Studies on the Distribution and Effects of Cyclophosphamide (NSC-26271) in Normal and Neoplastic Tissues 459
Peter J. Houghton, Kenneth D. Tew, and David M. Taylor
Institute of Cancer Research, Sutton, Surrey, England

Studies on the Enhancement by Cyclophosphamide (NSC-26271) of Artificial Lung Metastasis After Labeled Cell Inoculation 465
J. de Ruiter, T. Smink, and L. M. van Putten
Radiobiological Institute TNO, Rijswijk, The Netherlands

Distinctive Effects of Mechlorethamine (NSC-762), Cyclophosphamide (NSC-26271), and BCNU (NSC-409962) on Transcription in Ehrlich Cells 471
R. J. Rutman and N. G. Avadhani
University of Pennsylvania, Philadelphia

Aspects of the Teratology of Cyclophosphamide (NSC-26271) 477
R. Ashby, L. Davis, B. B. Dewhurst, R. Espinal, R. N. Penn, and D. G. Upshall
The Hatfield Polytechnic, Hertfordshire, England

The Use of Deuterated Analogs in Qualitative and Quantitative Investigations of the Metabolism of Cyclophosphamide (NSC-26271) 483
P. J. Cox, P. B. Farmer, A. B. Foster, E. D. Gilby, and M. Jarman
Institute of Cancer Research, London, England

Studies on the Disposition of Cyclophosphamide (NSC-26271) in Tumor-Bearing Mice by Means of Gas Chromatography-Chemical Ionization-Mass Fragmentography 493
C. Pantarotto, A. Martini, G. Belvedere, M. G. Donelli, and A. Frigerio
Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy

Qualitative and Quantitative Investigations of Cyclophosphamide (NSC-26271), Cyclophosphamide Metabolites, and Related Compounds by Field-Desorption Mass Spectrometry 501
H.-R. Schulten
Institute of Physical Chemistry, University of Bonn, West Germany

Characterization of Cyclophosphamide (NSC-26271) Metabolites and Related Derivatives by Field-Desorption and Electron-Impact Mass Spectrometry 509
M. Przybylski, H. Ringsdorf, G. Voelcker, U. Draeger, G. Peter, and H.-J. Hohorst
Institute of Organic Chemistry, University of Mainz, West Germany

X-Ray Crystallographic Studies of Cyclophosphamide (NSC-26271) Derivatives 517
Arthur Camerman, H. Warren Smith, and Norman Camerman
University of Washington, Seattle

Papers presented at meeting but published elsewhere:

Metabolites of Cyclophosphamide, 4-Hydroxycyclophosphamide, and Phosphoramide Mustard in Mouse Blood: A Time Study and Evidence for Phosphoramide Mustard as the Cytotoxic Metabolite
Robert F. Struck
Southern Research Institute, Birmingham, Alabama
Published in *Biomedical Mass Spectrometry* 2:46-52, 1975.

Stem-Cell Response of B16 Melanoma and Lewis Lung Carcinoma
G. G. Steel
Institute of Cancer Research, Sutton, Surrey, England
Published in *Cancer Research* 35:1530-1535, 1975; and "Cancer Medicine" (Holland JF, and Frei E III, eds), Philadelphia, Lea and Febiger, 1973.

Meeting Participants 523

The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Introduction

Cyclophosphamide is not only one of the most extensively used chemotherapeutic alkylating agents but it has also become one of the most intensively studied. The interest shown in this drug is primarily due to two factors: its broad spectrum of activity against human and animal tumors and its complex metabolic pathway. In order to generate antitumor activity, cyclophosphamide must undergo specific metabolism, the key step of which involves hydroxylation mainly, if not exclusively, in the liver. Although much detail has now been revealed of the subsequent profile of metabolism, the reasons for the comparative selectivity and effectiveness of cyclophosphamide remain obscure.

The aim of the Symposium on the Metabolism and Mechanism of Action of Cyclophosphamide held at the Chester Beatty Research Institute, London, England, July 10-12, 1975, was to consolidate current knowledge by bringing together for debate those workers active in this area of research. The Symposium Organizing Committee was gratified by the enthusiastic response to invitations to participate in the meeting and with the vigor of the discussion associated with papers presented.

Although the principal emphasis of the papers was on the metabolism of cyclophosphamide, relevant contributions were also made on isophosphamide and the toxicologic, immunologic, and teratologic effects of the drugs.

There is no doubt that the Symposium greatly enhanced the mutual appreciation of the work and research approaches of the participants. The Organizing Committee believes that not only will the Symposium have served as a stimulus to much new individual research but also that it will have promoted new collaborative projects.

The co-operation of the Editorial Board in permitting publication of the Proceedings in one issue of *Cancer Treatment Reports* is gratefully acknowledged. The views expressed by individual authors are not necessarily those of the guest editors or of the Journal.

The financial support of the Institute of Cancer Research and of Asta-Werke A. G. is gratefully acknowledged.

Peter J. Cox
Peter B. Farmer
Michael Jarman

Symposium Organizing Committee:
A. B. Foster, Chairman
P. B. Farmer, Secretary
T. A. Connors
P. J. Cox
M. Jarman

Comparative Pharmacologic Study In Vitro and In Vivo With Cyclophosphamide (NSC-26271), Cyclophosphamide Metabolites, and Plain Nitrogen Mustard Compounds[1,2]

N. Brock[3,4,*]

SUMMARY

This paper deals with the problem of the relative selectivity of the antitumor effect of cyclophosphamide (CP). CP and its metabolites are pharmacologically characterized by determining their chemical and biologic activities in vitro and their pharmacotherapeutic properties in vivo. Of particular importance is the specificity of the cytotoxic activity (cytostatic units/μmol) in vitro and the margin of safety (therapeutic index) in vivo. The pharmacologic data reveal: *a.* Of the various metabolites of CP only 4-hydroxycyclophosphamide, the primary activation product, exerts a highly specific cytotoxic activity in vitro and has a wide margin of safety in vivo. *b.* The decisive step in toxication is the formation of the alkylating *N,N*-bis(2-chloroethyl)phosphorodiamidic acid after acrolein has been split off.

[Cancer Treat Rep 60:301-307, 1976]

Before dealing with the subject of this paper, please allow me to say some introductory words about the history of cyclophosphamide (CP) and related oxazaphosphorine compounds.

Advancement in the field of cancer chemotherapy decisively depends on whether it is possible to develop agents with a higher selectivity of antitumor effects, thus obtaining a wider margin of safety, ie, a less risky therapy. This has been the leading principle in Asta-Werke cancer research for more than 20 years. At the suggestion of H. Druckrey, stilbestrol diphosphate was developed in 1952 for the treatment of metastasizing prostatic carcinoma (1), CP was developed in 1958 (2), trophosphamide and isophosphamide (IP) were developed in 1968 (3,4), and new types of mixed-function oxazaphosphorine oxides were developed in 1974. This latter group of compounds allows the induction of specific immunotolerance in warm-blooded animals (5,6).

"The study of CP has contributed very much to cancer research." With this sentence Dr. Hill from the Southern Research Institute, Birmingham, Ala, begins his excellent monograph "A Review of Cyclophosphamide," in which he describes the significant contributions of many investigators and scientists from all over the world (7). This world-wide interest in CP has led to many contacts and friendships for which I wish to express my cordial thanks.

The present symposium "Metabolism and Mechanism of Action of CP" promises to become a climax in the scientific treatment of the CP problems. Our colleagues of the Chester Beatty Research Institute, London, England, who have provided valuable contributions to our subject, have prepared this symposium with great care and skill. On behalf of all of us, I should like to sincerely thank these gentlemen for their efforts, and in full recognition of the high tradition of this Institute in the field of cancer research, I should also like to express our highest esteem for Sir Alexander Haddow, the Institute's former director, one of the most famous pioneers of cancer research.

ADAPTION OF THE TRANSPORT FORM/ACTIVE FORM PRINCIPLE IN NITROGEN MUSTARD COMPOUNDS

The development of CP was based on the idea of converting the highly toxic agent mechlorethamine

[1]Supported by Bundesministerium für Forschung und Technologie.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Department of Pharmacology, Asta-Werke Aktiengesellschaft, Chemische Fabrik, Bielefeld, West Germany.

[4]I wish to acknowledge the following people who assisted me with the work reported in this manuscript: Drs. Arnold (deceased), Bourseaux, Bekel, Druckrey, Hohorst, Potel, Schneider, Wilmanns (deceased), and Burkert; also J. Kottmeier, M. Elliger, and P. Licher.

*Reprint requests to: Prof. N. Brock, Asta-Werke Aktiengesellschaft, Chemische Fabrik, D-4800 Bielefeld 14 Postfach 14 01 29, West Germany.

into an inert transport form, from which only the active form would be released in the body. For such a conversion Harper (8) proposed the term "drug latentiation." With the realization of this idea it was indeed possible to considerably reduce the general toxicity of the drug while its chemotherapeutic potency was preserved, thus obtaining a greater tumor selectivity and a wider margin of safety (9,10).

The transport form is defined pharmacologically as an almost inert compound which before exerting its biologic activity has to be converted by a metabolic process or at least by an enzymatically catalyzed reaction. The active form is defined as the compound which spontaneously reacts directly with the biologic substrate. Between the transport and the active form several intermediate products may be formed whose structures should be elucidated since the pharmacologic characterization is of great importance for understanding the drug's metabolic changes and its mechanism of action.

The process involved was more complex than we had expected on hypothetical grounds. Although in view of our present knowledge the transport form/active form principle had its heuristic value for the development of CP, it did not permit full elucidation of the problem of specificity. Nevertheless, it has been useful in this respect insofar as it provided a stimulating tension between working hypothesis and experimental findings.

Pharmacologic Characterization of Nitrogen Mustard Oxazaphosphorines

With the development of CP a new reaction type was created, whose chemical and pharmacologic behavior was distinctly different from that of the nitrogen mustards previously known. The latter were either chemically *and* pharmacologically highly active or, like the acyl derivatives of *nor*-nitrogen mustard synthesized by Ross and Preussmann (11,12), they were chemically *and* pharmacologically practically inert. In contrast, CP shows only low chemical reactivity in vitro and correspondingly only slight biologic reactivity, but it has a high cancerotoxic activity in vivo. At the same time its general toxicity is considerably reduced and its margin of safety is widened.

For the determination of these different properties, the pharmacologic screening has to be adjusted to the different reaction types by developing suitable tests for the assessment of the drug's chemical and pharmacologic activities in vitro and in vivo.

In view of the significance of pharmacologic assessment, not only for the classification of the individual agents but also for the characterization of their individual metabolites, these tests will be described briefly:

a. The chemical reactivity of the nitrogen mustard compounds can be assessed by their behavior in aqueous solution where the rate of chloride ion liberation from the 2-chloroethyl group is taken as a yardstick for chemical reactivity. In CP the ionization rate is considerably reduced in comparison to the parent compounds (fig 1). In addition, the chemical reactivity of a nitrogen mustard compound can be assessed by means of the 4-(4'-nitrobenzyl)pyridine (NBP) test. This reagent allows quantitative determination of the actual alkylating potency of a compound. In contrast to the alkylators of high reactivity, CP does not react directly with NBP, but only after previous hydrolysis.

b. The cytotoxic potency of a nitrogen mustard compound is assessed in an incubation test, in which cancer cells of a known chemoresistance in vitro are incubated with increasing concentrations of the test agent. After washing the compound out of the solution, the cells' transplantability to untreated animals is tested. Determination of the 50% effective concentration (EC50) allows the quantitative assessment of the actual cytostatic activity of a compound even in the presence of potentially active transport forms. Such a study is an adequate complement to the above-described chemical tests which we invariably used parallel to them, so as to characterize the individual compounds and their metabolites, relating them of course to a standard agent. In contrast to other nitrogen mus-

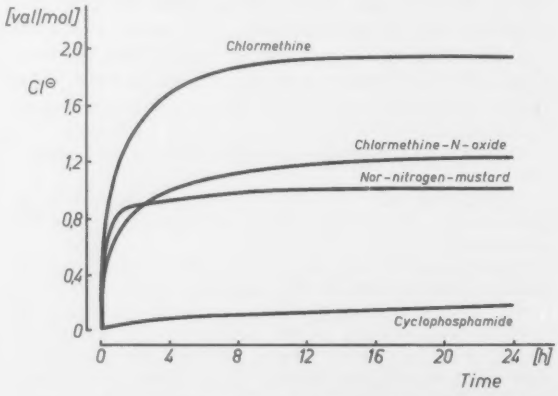


FIGURE 1.—Chloride ion liberation of chlormethine, chlormethine-*N*-oxide, *nor*-nitrogen mustard, and CP (bicarbonate buffer solution pH 7.5, 0.026 M, 37.5°C).

tards, CP does not exert any effect in vitro in very high concentrations (table 1).

c. The therapeutic value of nitrogen mustard compounds is assessed in adequate chemotherapy tests in the intact animal. The quantitative determination of the various chemotherapeutic and toxic actions is effected by establishing the dose-action regression lines. The curative action is assessed in a number of tumor models, especially in the rat, the biologic properties of which (eg, chemosensitivity) are well known and continuously controlled. For the so-called transport form, the assessment of its cancerotoxic potency in vivo serves as proof of its intracorporeal conversion into the active form, and thus a kind of predecision on the principles of its mechanism of action. Table 2 shows the high activity of CP in the Yoshida ascitic sarcoma of the rat. In comparison to other alkylating agents, its therapeutic index is up to ten times higher.

Interrelation Between Chemical Constitution and Biologic Activity in the Series of Nitrogen Mustard Oxazaphosphorines

With the aid of the above-mentioned methods the various types of nitrogen mustard oxazaphosphorines were clearly defined and the individual compounds were assigned to one of the reaction types described. Since some structural peculiarities, which were published in detail in 1961 (13), are also important for the elucidation of the CP metabolism, they will be briefly described.

A graph where the chloride ion liberation after 24 hours is plotted on the abscissa (bicarbonate buffer solution, pH 7.5, 37.5°C) and the D50 index (LD50/CD50, 50% lethal dose/50% curative dose) is plotted on the ordinate, clearly shows the different properties of the three reaction types (fig 2). Two groups of points are marked out near the abscissa. These compounds have only low indices and they are absolutely different in their chemical reactivity. The right-hand group of compounds has a high chemical reactivity and the left-hand group has a low one. The third group of points with the compounds CP, B 717, B 699, B 576, as well as trophosphamide and IP differs distinctly from the former two series. With indices between nine and 24, as an expression of their considerably wider margin of safety, they are definitely superior to about 800 compounds.

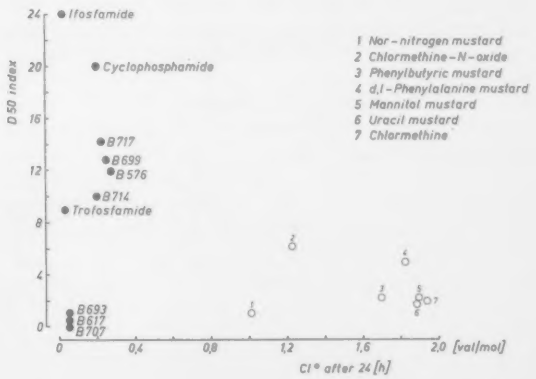


FIGURE 2.—Relationship between chemotherapeutic effectiveness and chemical reactivity of some nitrogen mustard oxazaphosphorines compared with various highly reactive nitrogen mustard compounds.

TABLE 1.—Cytotoxic activity of chlormethine, chlormethine-*N*-oxide, and *nor*-nitrogen mustard compared with CP

Agent	Yoshida ascites sarcoma in vitro (37°C, 1 hr): EC50 (μg/ml)
Chlormethine	0.1
Chlormethine-*N*-oxide	0.5
Nor-nitrogen mustard	1.0
CP	>3000

TABLE 2.—Indices of the curative and lethal effect of CP compared with chlormethine-*N*-oxide and *nor*-nitrogen mustard by iv administration to Yoshida ascites sarcoma of the rat

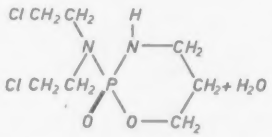
Agent	LD50 (mg/kg)	Yoshida ascites sarcoma		
		CD50 (mg/kg)	CD50 % of LD50	Therapeutic index (LD5/CD95)
CP	160	4.5	2.8	8.5
Chlormethine-*N*-oxide	50	5.4	10.9	2.2
Nor-nitrogen mustard	100	40	40	0.65

The greater tumor selectivity of this group of compounds is due to a certain chemical structure: chemically they are diamido-mono-esters of nitrogen mustard phosphoric acid which, like CP, trophosphamide, and IP, have the amide-linked *N*-propylene bridge cyclically arranged as shown in figure 3. For this reaction type, the *N*-propylene group is important. Even slight changes in the chemical structure alter the specific mechanism of action and make the characteristic selectivity of tumor action ineffective.

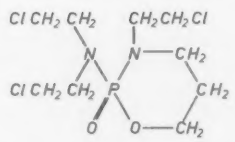
Especially important is the methyl substitution in the six-membered heterocyclic ring which I will describe briefly. As the indices in table 3 show, the derivatives of CP mono-methylated in positions 4, 5, and 6 of the ring still possess the specific character of the parent compound. The D50 indices lie between 12 and 14.4.

Of even greater interest is the behavior of the di-substituted homologs, particularly after dimethyl substitution at carbon atoms 4, 5, or 6 of the ring. Dimethyl substitution of carbon atoms 4 and 5 means a complete loss of the biologic activity, especially the curative one, whereas dimethyl substitution at carbon atom 6 does not affect the specific qualities of the parent compound (table 4).

Cyclophosphamide



Trofosfamide



Ifosfamide

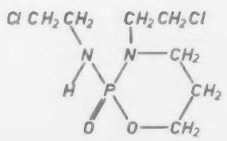
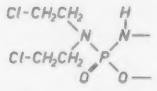


FIGURE 3.—Structures of CP, trophosphamide, and IP.

TABLE 3.—Monomethylation at the carbon atoms of the oxazaphosphorine ring of CP (comparison of the D50 indices)



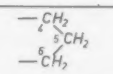
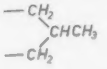
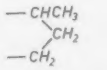
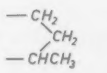
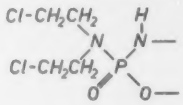
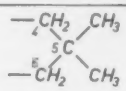
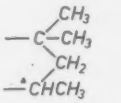
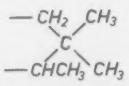
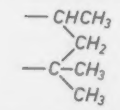
Agent		LD50(1)/CD50(4)
CP		20.0
B 717		14.4
B 699		12.5
B 576		12.0

TABLE 4.—Dimethylation at the carbon atoms of the oxazaphosphorine ring of CP (comparison of the D50 indices)



Agent		LD50(1)/CD50(4)
B 707		< 0.1
B 617		0.5
B 693		1.0
B 714		10.0

Whereas 15 years ago these characteristic differences could not be interpreted, recent findings concerning CP metabolism have not only elucidated the behavior of the mono-methyl compounds, but also di-methylation at a carbon atom of the

ring. Dimethyl substitution at carbon atom 4 prevents hydroxylation at this position. According to Cox et al (14), the introduction of two methyl groups at carbon atom 5 inhibits the release of an unsaturated aldehyde (in analogy to the acrolein formation on the cleavage of CP), and thus the toxication into the alkylating nitrogen mustard phosphoric ester diamide.

THE PROBLEM OF SELECTIVITY OF CP

Metabolism of CP in Warm-Blooded Animals

The problem of the relative selectivity of the cancerotoxic action of CP has been intensively studied in the past 15 years. In 1962, together with Hohorst (15,16), we showed that the conversion of CP into cytostatically active metabolites preferentially occurs in the liver and, to a considerably smaller extent, in the lungs, and that the presence of oxygen is a necessary prerequisite for this activation. In addition, studies on isolated-cell fractions have shown the activation process to be catalyzed by the endoplasmic reticulum or the so-called microsomal fraction of the liver in the presence of oxygen and $NADPH_2$ or a $NADPH_2$-regenerating system. Thus, this process appears to be what has been called a mixed-function hydroxylation of CP. The chemistry of the metabolic breakdown in vivo has been elucidated in many individual stages (fig 4). After administration of CP, the following metabolites have been found in the bodies of warm-blooded animals: 4-hydroxycyclophosphamide (4-hydroxy-CP), 4-ketocyclophosphamide (4-keto-CP), aldophosphamide, *N,N*-bis(2-chloroethyl)phosphorodiamidic acid, acrolein, carboxyphosphamide, and *nor*-nitrogen mustard.

The main difficulty in clarifying the metabolism of CP was not particularly that of a theoretical design of a number of breakdown schemes, but rather the corroborative assays, isolation, and identification of the chemical structure of the intracorporeally formed metabolites as well as their synthesis. Only after a sufficient quantity of the various metabolites was available in pure form did the pharmacologic characterization and evaluation become possible.

Special efforts were necessary for the chemical synthesis of the primary activated metabolites, the tautomeric compounds 4-hydroxy-CP and aldophosphamide. Takamizawa et al (17) have succeeded in synthesizing 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP), the deoxidation of which has permitted the preparation of 4-hydroxy-CP. However, we have not yet been able to obtain aldophosphamide in pure form. Voelcker et al (18) have shown that a balance exists between 4-hydroxy-CP and aldophosphamide which they studied in detail.

Pharmacologic Characterization of CP Metabolites

At this point we should become acquainted with the results of the pharmacologic assays. In addition to the data on CP and its metabolites, table 5 contains data of two more nitrogen mustard compounds, chlormethine and chlormethine-*N*-oxide. The methods of testing have been described in detail at the beginning of this paper. Particular attention should be paid to the column in which the

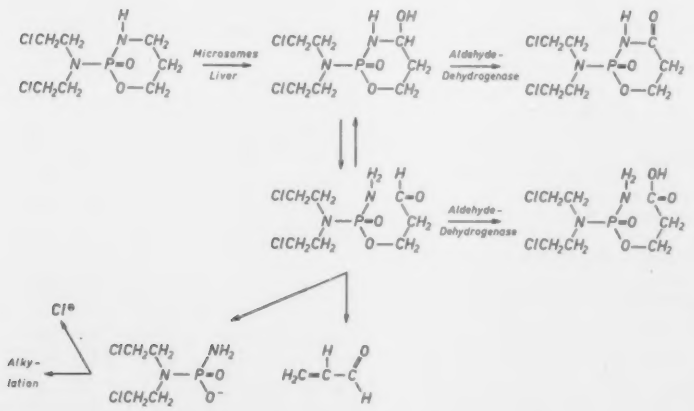
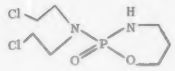
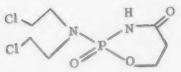
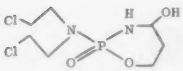
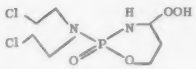
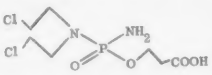
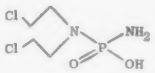
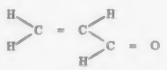
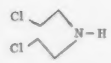
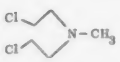


FIGURE 4.—Activation of CP in the liver.

TABLE 5.—Chemical reactivity and biologic activity of nitrogen mustard compounds and CP metabolites

Nitrogen mustard derivatives		Molecular weight	In vitro			In vivo		
			Alkylating activity* (%)	Chloride ion hydrolysis: 1 val/mol† in—	Cytotoxic activity‡ (CU/ μmol)	CD50‡ (mg/kg)	LD50 (mg/kg)	D50 index (LD50/CD50)
CP		279§	1.3	> 7 days	< 0.03	1.25	220	175
4-Keto-CP		275.1	1.2	> 7 days	< 0.07	> 800	> 800	—
4-Hydroxy-CP		277.1	65	480 mins	63	1.25	150	120
4-Hydroperoxy-CP		293	40	240 mins	21	1.25	97.5	78
Carboxyphosphamide		293	85	> 7 days	0.1	200	~800	~4
N,N-Bis-(2-chloroethyl)phosphorodiamidic acid		221	90	60 mins	2.5	20	61	3.5
Acrolein		56.06	(1.9)	—	0.4	> 2.15	7.3	—
Nor-nitrogen mustard		178.5	100	60 mins	1.35	40	100	2.5
Chlormethine		192.5	100	15 mins	138	0.25	1.1	4.4
Chlormethine-*N*-oxide		208.6	100	180 mins	1.5	5	60	12

*NBP test.
†pH 7.5; 37°C.
‡Yoshida sarcoma.
§Monohydrate.

cytostatic activity, expressed as cytostatic units (CU) per μmol of alkylating agent, is quantitatively indicated. Together with the therapeutic index, this quotient indicates the principle of selectivity of the cancerotoxic effect. All three nitrogen mustard compounds tested have a high chemical reactivity in vitro, which can be measured by their alkylating activity in the NBP test and the rate of chloride ion liberation. The cytotoxic activity of chlormethine with a quotient of 138 is strikingly high and superior to all standard preparations to which the compounds were compared. However, the margins of safety of chlormethine and the other two nitrogen mustard compounds are relatively narrow. The cyclic compounds CP and 4-keto-CP are both inert in vitro; even their cytotoxic activity is practically nil. However, in vivo they show a decisive difference. As a chemotherapeutic agent CP is highly active, the relative selectivity of its antitumor effect being expressed by the high D50 index of 175, whereas 4-keto-CP is practically inert even in vivo. The latter compound cannot be activated or metabolized further in the body.

The two compounds oxidized in the carbon 4 position, the primary activated metabolite 4-hydroxy-CP and the synthetic 4-hydroperoxy-CP, do not show the same in vitro reactivity with regard to the chloride ion liberation as the plain nitrogen mustards; on the other hand they are not as stable as CP or 4-keto-CP. Even with regard to their alkylating activity they hold an intermediate position. Their cytotoxic activity is really outstanding. With an index of 63, 4-hydroxy-CP is considerably superior to all standard agents, exceeded only by chlormethine. In contrast to chlormethine, 4-hydroxy-CP has about the same chemotherapeutic effect but only a relatively low toxicity in vivo, and thus a considerably greater therapeutic index than the former (the D50 index was 120). The values for 4-hydroperoxy-CP are similar but are below those of 4-hydroxy-CP. *N,N*-bis(2-chloroethyl)-phosphorodiamidic acid shows basically the same behavior as the plain nitrogen mustard compounds chlormethine-*N*-oxide and *nor*-nitrogen mustard. In spite of its intense alkylating activity, its cytotoxic activity is only moderate (2.5 CU/μmol), ie, only one twenty-fifth that of 4-hydroxy-CP. The margin of safety of this compound is relatively small.

Carboxyphosphamide, an open-ring compound, is hardly active at all, either in vitro or in vivo.

Under our test conditions, acrolein does not show any effect which might be of consequence for the problem of selectivity. On the basis of these data, we can define the pharmacotherapeutic properties of the various CP metabolites and elucidate their significance for tumor-action selectivity. In another paper presented at this meeting (19), Hohorst et al gave a survey of the various biochemical, pharmacotherapeutic, and pharmacokinetic results. Therefore, I shall confine myself to outlining some characteristics which directly result from the pharmacologic data.

1. Of the various metabolites of CP only 4-hydroxy-CP has the same characteristic selectivity of antitumor effect in vivo as CP. The relative tumor selectivity is obviously due to the presence of an oxazaphosphorine ring with three carbon atoms in the ring.

2. With regard to their cytotoxic activity in vitro, there are great differences between the various alkylators. Of the CP metabolites under test, only 4-hydroxy-CP exerts a highly specific cytotoxic activity. All other metabolites, nitrogen mustard phosphoric acid diamide, carboxyphosphamide, and *nor*-nitrogen mustard in particular, do not possess this characteristic specificity.

3. The tautomeric compounds 4-hydroxy-CP and aldophosphamide have a weak alkylating activity; only after cleavage of the phosphoric ester bond do they acquire strong alkylating properties (18). Therefore, we consider them to be transport forms of higher reactivity than CP. The decisive step in the toxication process is the formation of the alkylating agent nitrogen mustard phosphoric acid diamide. Hohorst et al (19) describe the role of this compound in CP kinetics.

REFERENCES

1. Druckrey H. Experimentelle Grundlagen der Chemotherapie des Krebses. Dtsch Med Wochenschr 77:1534, 1952.
2. Arnold H, Bourseaux F, and Brock N. Neuartige Krebs-Chemotherapeutika aus der Gruppe der zyklischen *N*-Lost-Phosphamidester. Naturwissenschaften 45:64, 1958.
3. Brock N. Pharmacological studies with ifosfamide, a new oxazaphosphorine compound. *In* Advances in Antimicrobial and Antineoplastic Chemotherapy. Proceedings of the 7th International Congress of Chemotherapy, Prague, 1971. Munich, Urban & Schwarzenberg, 1972.
4. ———. Pharmakologische Untersuchungen mit Trofosfamid (Ixoten), einem neuen Oxazaphosphorinoxid. Med Monatsschr 27:390-394, 1973.
5. Brock N, and Kuhlmann J. Pharmacological studies with alkylsulfonyloxyalkyl substituted and chloroethyl substituted oxazaphosphorine-2-oxides. 1. Communication: relationship between chemical structure and pharmacological action. Arzneim Forsch 24:1139-1149, 1974.
6. Brock N, and Potel J. Pharmakologische Untersuchungen mit Alkylsulfonyloxyalkyl-bzw. Chloräthyl-substituierten Oxazaphosphorin-2-oxiden. 2. Mitteilung: Pharmakologische Charakterisierung von 2-Alkyl-sulfonyl-oxyalkylamino-oxazaphosphorin-2-oxiden unter Einbeziehung immunologischer Reaktionen. Arzneim Forsch 24:1149-1160, 1974.

7. Hill DL. A Review of Cyclophosphamide. Springfield, Charles C. Thomas, 1975.
8. Harper NJ. Drug latentiation. J Med Pharm Chem 1:467-500, 1959.
9. Brock N. Zur pharmakologischen Charakterisierung zyklischer *N*-Lost-Phosphamidester als Krebs-Chemotherapeutika. Arzneim Forsch 8:1, 1958.
10. Brock N, and Wilmanns H. Wirkung eines zyklischen *N*-Lost-Phosphamidesters auf experimentell erzeugte Tumoren der Ratte. Dtsch Med Wochenschr 83:453, 1958.
11. Preussmann R. Chlor-Abspaltbarkeit und Wirksamkeit von *N*-Lost-Verbindungen. Arzneim Forsch 8:9-10, 1958.
12. Ross WCJ. The chemistry of cytotoxic alkylating agents. Adv Cancer Res 1:397-449, 1953.
13. Arnold H, Bourseaux F, and Brock N. Über Beziehungen zwischen chemischer Konstitution und cancerotoxischer Wirkung in der Reihe der Phosphamidester des Bis-(β-chloräthyl)-amins. [Relationship between chemical constitution and carcinotoxic activity of the phosphamide esters of bis(β-chloroethyl)amine.] Arzneim Forsch 11:143-158, 1961.
14. Cox PJ, Farmer PB, and Jarman M. The use of cyclophosphamide analogues in mechanistic studies of the metabolism of cyclophosphamide. *In* Advances in Mass Spectrometry in Biochemistry and Medicine (Frigerio A, and Castagnoli N, eds). New York, Spectrum Publications, 1975. In press.
15. Brock N, and Hohorst H-J. Über die Aktivierung von Cyclophosphamid im Warmblüterorganismus. [Activation of cyclophosphamide in the warm blooded organism.] Naturwissenschaften 49:610-611, 1962.
16. ———. Über die Aktivierung von Cyclophosphamid in vivo und in vitro. [Activation of cyclophosphamide in vivo and in vitro.] Arzneim Forsch 13:1021-1031, 1963.
17. Takamizawa A, Matsumoto S, Iwata T, et al. Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
18. Voelcker G, Draeger U, Peter G, et al. Studien zum Spontanzerfall von 4-Hydroxycyclophosphamid und 4-Hydroperoxycyclophosphamid mit Hilfe der Dünnschichtchromatographie. Arzneim Forsch 24:1172-1176, 1974.
19. Hohorst H-J, Draeger U, Peter G, et al. The problem of oncostatic specificity of cyclophosphamide (NSC-26271): studies on reactions that control the alkylating and cytotoxic activity. Cancer Treat Rep 60:309-315, 1976.

The Problem of Oncostatic Specificity of Cyclophosphamide (NSC-26271): Studies on Reactions that Control the Alkylating and Cytotoxic Activity[1,2]

H.-J. Hohorst,* U. Draeger, G. Peter, and G. Voelcker[3,4]

SUMMARY

The relatively high oncostatic specificity of cyclophosphamide (CP) in vivo is shown to be due to the cytotoxic specificity of 4-hydroxycyclophosphamide (4-hydroxy-CP), the first product of metabolic activation of CP in the liver. This specificity can be evaluated not only in vivo by measuring the therapeutic index, but also in vitro by determining its cytotoxicity against Yoshida ascites tumor cells. Evidence is given that 4-hydroxy-CP is not an alkylating agent itself, but attains this property only by release of an alkylating *N,N*-(2-chloroethyl)phosphorodiamic acid moiety and acrolein. The energetic source for this rate-limiting toxication results from the resonance stabilization of the released acrolein. Reactions at the cryptoaldehyde group of 4-hydroxy-CP, which reduce or prevent the resonance stabilization of the 3-carbon unit to be released, lead to a deactivation of the primary metabolite of CP thus reducing or even preventing toxication, and hence influencing both the alkylating and cytotoxic activities of the molecule. Accordingly, it could be demonstrated by the reaction of 4-hydroxy-CP with thiols yielding 4-(*S-R*)-mercapto CP derivatives that the toxication of 4-hydroxy-CP can be controlled under physiologic conditions of pH and temperature. In the case of free protein sulfhydryl groups, this reaction also leads to fixation onto a macromolecule of the CP metabolite. On the basis of these peculiar reactivities of the oxazaphosphorine ring of 4-hydroxy-CP and of the partial reaction kinetics involved during toxication or deactivation, the significance of these findings to the problem of CP specificity is discussed.

[Cancer Treat Rep 60:309-315, 1976]

Cyclophosphamide (CP) plays an important role in both clinical therapy and experimental laboratory work because of its relatively high oncostatic specificity. This oncostatic specificity, which can be evaluated quantitatively in tumor-bearing laboratory animals by determining the therapeutic index (TI), was first demonstrated by Brock (1) and Brock and Wilmanns (2) and has been the leading criterion for the design of CP and its analogs in the Asta-Werke Laboratories. However, the question of which peculiar property of CP or its metabolites is responsible for the oncostatic specificity has so far remained uncertain. Early assumptions that CP specificity could result from differences between the release of alkylating moieties in tumor and nontumor cells due to different levels of phosphoramidases (3) could not be demonstrated and became unlikely when the activation of CP to cytotoxic metabolites was shown to occur mainly in the liver (4). More recently, a new hypothesis has been advanced by Sladek (5) and Connors et al (6) who postulate different balances between the accumulation of 4-hydroxycyclophosphamide (4-hydroxy-CP) or its tautomer aldophosphamide and enzymatic

[1]Supported by Bundesministerium für Forschung und Technologie.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany.

[4]We thank Miss Anke Wilkoewitz and Mrs. Xenia Klikic for technical assistance. The collaboration and continuing interest of Dr. N. Brock, Asta-Werke, Bielefeld, Germany, are gratefully acknowledged.

**Reprint requests to:* Dr. H.-J. Hohorst, Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, Theodor-Stern-Kai 7, 6000 Frankfurt, 70 West Germany.

deactivation to nontoxic 4-ketocyclophosphamide (4-keto-CP) or the less toxic carboxyphosphamide, which should lead to different levels of cytostatically active metabolites in tumor cells and other tissues. Evidently, more experimental work must be done on this interesting hypothesis. However, some doubts exist as to whether the phenomenon of CP specificity could be explained in this manner.

In this paper we shall deal with studies on peculiar properties and reactions of 4-hydroxy-CP which may be relevant for the oncostatic specificity of CP. The details of the pharmacokinetics of 4-hydroxy-CP and other CP metabolites in vivo, the permeation kinetics of CP metabolites into tumor cells, and the special reactions of 4-hydroxy-CP with thiols will be discussed in other reports in this issue (7-10).

MATERIALS AND METHODS

CP, phosphoramide mustard, *nor*-nitrogen mustard, cytoxylamine, and aldophosphamide-diethylacetal were gifts of Asta-Werke AG, Bielefeld, Germany. (We are indebted to Prof. Brock, Dr. Bourseaux, and Dr. Scheffler for generous gifts of these substances.) 4-Hydroxy-CP was prepared by reduction of 4-hydroperoxy-CP with triphenylphosphine in CH_2Cl_2 as previously reported (11,12). The melting point after recrystallization in CH_2Cl_2 was 52°C-53°C. All other substances and materials were obtained from commercial sources.

Alkylating activity and alkylating capacity.—Alkylating substances were determined by means of the 4-(4-nitrobenzyl)pyridine (NBP) test of Friedman and Boger (13) after acid hydrolysis in 1 N HCl at 100°C for 10 minutes. The total alkylating and potential alkylating material in solution thus determined was designated the *alkylating capacity* of the sample. The *alkylating activity* of the substance or sample tested in aqueous solution at pH 7 (37°C) was measured by determining the rate of decrease of the alkylating capacity by means of the NBP test after acid hydrolysis. The alkylating activity determined in this manner is the alkylating activity against water or the rate of hydrolysis of one 2-chloroethyl group of a *nor*-nitrogen mustard moiety.

Thin-layer chromatography (TLC).—Separation of CP and CP metabolites was performed by TLC on 1,3-propanediol-impregnated cellulose sheets with ethyl acetate as a mobile phase at 3°C-5°C, as described previously by us (12). Alkylating material on cellulose sheets was identified by means of an NBP spray (12). For quantitative determinations of alkylating metabolites after TLC, metabolite-containing zones were scraped off the cellulose sheet and eluted with aqueous or alcoholic solvent.

Assay for cytotoxicity in vitro.—To determine the cytotoxicity in vitro, Yoshida ascites tumor cells were incubated with increasing concentrations of the substance to be tested in Ringer hydrogen carbonate/glucose buffer according to Schmähl and Druckrey (14). After 2 hours' incubation at 37°C the tumor cells were washed and defined portions were injected in rats (for details see ref 4). One cytostatic unit (CU) is that amount of a cytostatically effective compound which produces survival in 50% of the animals after inoculation with tumor cells under the conditions of the test described above (4).

Therapeutic index (TI).—The oncostatic specificity of a compound was evaluated by determining the LD50/CD50 ratio (50% lethal dose/50% curative dose) with Yoshida ascites sarcoma-bearing rats according to Brock and Schneider (15). Both the in vitro assays for cytotoxicity and the TI determinations were performed in the laboratory of Dr. Brock.

RESULTS

Cytotoxic and Oncostatic Specificity of CP Metabolites in the Course of Metabolic Activation and Toxication

The metabolism of CP which yields the alkylating and cytotoxic phosphoramide mustard has two rate-limiting steps that are linked with characteristic alterations in both the chemical and biologic properties of the CP metabolites (fig 1). First, the activation of CP by microsomal hydroxylation (4) yields 4-hydroxy-CP, which equilibrates with its ring-opened tautomeric aldophosphamide (12). As shown in figure 1, 4-hydroxy-CP, and presumably aldophosphamide, exhibit a high cytotoxic activity in vitro against Yoshida ascites tumor cells and also a high TI when applied in vivo to tumor-bearing rats as shown by Brock (16). The cytotoxic activity of aldophosphamide could not be determined separately due to its fast equilibration with 4-hydroxy-CP (12). Furthermore, it can be seen that the TIs of CP and 4-hydroxy-CP are nearly equally high. However, the apparent alkylating activity of the primary CP metabolite was determined to be only relatively small (see also fig 3).

During the second rate-limiting step of the metabolic breakdown of CP, designated as toxication, the apparent alkylating activity of the reaction product phosphoramide mustard is raised while both the apparent cytotoxic activity in vitro and the TI in vivo decline strongly when compared with

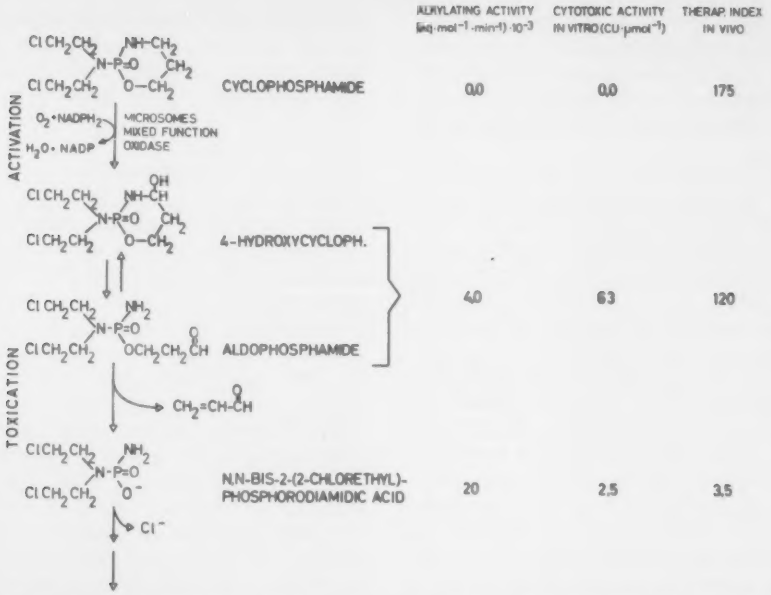


FIGURE 1.—Metabolic activation and toxication of CP. Apparent alkylating activities were measured in phosphate buffer (0.07 M, pH 7, 37°C). Cytotoxic activities on Yoshida ascites cells and TI values on Yoshida sarcoma-bearing rats from data of Brock (16) (*see* Materials and Methods). Values for 4-hydroxy-CP and aldophosphamide refer to an equilibrium of both tautomers.

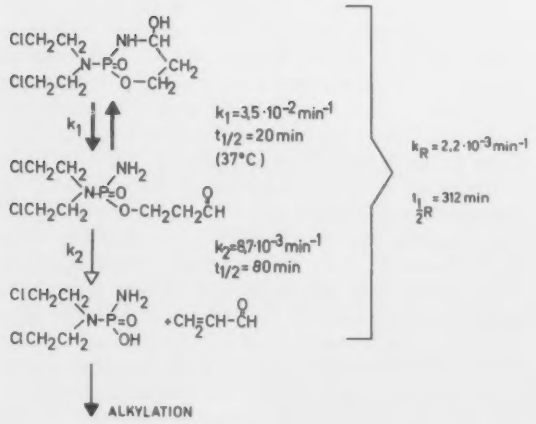


FIGURE 2.—Kinetics of breakdown of 4-hydroxy-CP. Kinetic constants were evaluated for first-order kinetics from decrease of alkylating capacity in phosphate buffer (0.07 M, pH 7, 37°C) by means of quantitative TLC (12). k_R and $t_{1/2R}$ were calculated from acrolein release in Ringer phosphate buffer containing 1.8 mM Ca^{++} ions (12).

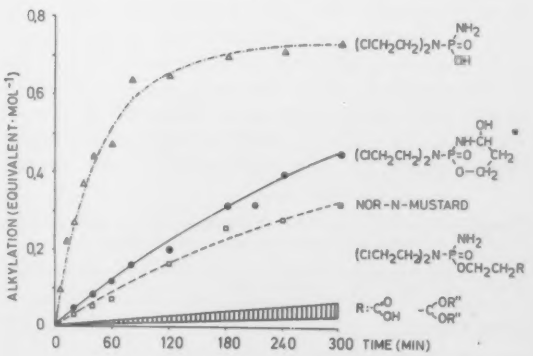


FIGURE 3.—Alkylation rates of *N,N*-bis(2-chloroethyl)-phosphoramide derivatives in 0.07 M phosphate buffer (pH 7, 37°C). The alkylation rate was measured by determining the decrease of alkylating capacity of the substances indicated by *. For 4-hydroxy-CP the alkylation rate was determined in Ringer phosphate buffer containing 1.8 mM Ca^{++} ions (*see* Materials and Methods).

4-hydroxy-CP. From these data one may conclude that the oncostatic specificity of CP in vivo, as measured by the TI, is due to the oncostatic specificity of the primary activated metabolite (16) but is, however, not directly related to the alkylating activity.

Because of the coincidence of the decay of cytotoxicity in vitro and the oncostatic specificity in vivo that can be seen after toxication, one may ask whether the in vitro cytotoxicity may be related to the in vivo specificity of CP, ie, the TI. The available data point to such a correlation between these parameters as will be shown in table 1, where the in vitro cytotoxicities of some nitrogen mustards, including CP metabolites, are compared to the oncostatic specificity in vivo. Since for chemical reasons one can assume that all nitrogen mustards exert their cytotoxic action only by means of alkylations of nucleophilic target molecules in the cells, the cytotoxicity in vitro should depend mostly on the alkylating activity of the compounds under physiologic conditions of pH and temperature unless physicochemical factors, such as permeability of the drugs into cells or solubility, are predominant. Thus, if one relates the in vitro cytotoxicity of nitrogen mustards to their alkylating activity, designated here as cytotoxic specificity in vitro, nearly the same values are expected for all compounds studied. However, as shown in table 1, this is not the case. 4-Hydroxy-CP alone exceeds by far the cytotoxic specificity of the other nitrogen mustards. Furthermore, the parameter that governs the oncostatic specificity in vivo exhibits the same exceedingly high value only in the case of 4-hydroxy-CP. Indeed, these data suggest that some common additional factor is responsible for both the high cytotoxic specificity in vitro and the oncostatic specificity in vivo of 4-hydroxy-CP. This, however, has considerable consequences for the experimental work on CP specificity, since to determine the molecular basis for CP specificity, one can work with an in vitro assay system on tumor cells in suspension, thus excluding most of the burdensome in vivo problems. In any case, without regard to the correctness of these considerations, any hypothesis concerning the mechanism of the oncostatic specificity of 4-hydroxy-CP in vivo must also explain the high cytotoxic specificity of this compound in vitro.

Finally, from the data shown in figure 1 and table 1 it can be determined that neither phosphoramide mustard or *nor*-nitrogen mustard could bear the oncostatic specificity of CP in vivo as has been postulated recently by some authors (17,18). This has been previously ruled out by us (19) by the finding that the in vitro cytotoxicity of rat serum after CP administration, when referred to the total concentration of alkylating substances in the serum, was 32 CU/μmol of alkylating material. This is nearly as high as the value found for activated CP alone. Moreover, a detailed study which demonstrates relatively high levels of 4-hydroxy-CP as compared to low levels of phosphoramide mustard and *nor*-nitrogen mustard after administration of CP to small laboratory animals is discussed in another paper presented at this meeting (7).

TABLE 1.—Cytotoxic specificity of nitrogen mustard derivatives in vitro and oncostatic specificity in vivo

Compound	Cytotoxic specificity in vitro*	TI† (LD50/CD50)
4-Hydroxy-CP	160	120
Phosphoramide mustard	1.3	3.5
Nor-nitrogen mustard	4.1	2.5
N-oxid-mustard	3.6	12
Nitrogen mustard	12	4.4

*Calculated as the ratio of cytotoxic activity to alkylating activity × 10 from cytotoxic activity data of Brock (16) and the alkylating activities (*nor*-nitrogen mustard equivalent × mol^{-1} × min^{-1}) in phosphate buffer (0.07 M, pH 7, 37°C).

†TI data from Brock (16).

Peculiar Reactions of the Activated Metabolite(s) of CP: Deactivation and Toxication

By the microsomal activation of CP in the liver a cryptoaldehyde group is introduced at carbon 4 of the oxazaphosphorine ring of CP. This enables 4-hydroxy-CP to proceed through the rate-limiting toxication reaction to phosphoramide mustard, which has been suggested to be the ultimate alkylating product of CP metabolism (6). The half-life of the spontaneous breakdown of 4-hydroxy-CP in phosphate buffer under physiologic conditions of pH and temperature was found to be relatively long (fig 1) and could even be increased when the phosphate buffer was exchanged by Ringer phosphate buffer, possibly due to the presence of Ca^{++} ions (12). This phenomenon must be studied more intensively. Other possible ways to modulate the rate of the toxication step result from chemical reactions with the cryptoaldehyde group on carbon 4 of the oxazaphosphorine ring of 4-hydroxy-CP or the aldehyde group of aldophosphamide. Thus, dehydrogenation of 4-hydroxy-CP to 4-keto-CP or of aldophosphamide to carboxyphosphamide, and also nonenzymic reactions such as formation of hydrazones or acetalization of aldophosphamide, strongly reduce the toxication rate, and therefore the alkylating reactivity of the

products (fig 3) is also reduced. Furthermore, all these deactivated products of reactions on carbon 4 have little or no cytotoxicity as was first shown for 4-keto-CP (20). This phenomenon can only be understood if one assumes that all CP metabolites or derivatives containing an oxazaphosphorine ring or at least an intact phosphorus ester bond are not alkylating agents under physiologic conditions of pH and temperature and consequently are also not cytotoxic per se. Obviously, only after the release of a 3-carbon unit from these compounds in the form of acrolein (21) or the release of acrylic acid during the toxication step are the alkylating and cytotoxic activities attained. This probably indicates that by means of resonance stabilization, acrolein meets the energetic demands of the toxication reaction. Thus, when resonance stabilization cannot take place or is reduced, toxication will also be prevented or reduced.

Under in vivo conditions the enzymic dehydrogenation of 4-hydroxy-CP and aldophosphamide to yield nontoxic or less toxic 4-keto-CP and carboxyphosphamide respectively has been shown to take place (fig 4). Since the catalyzing enzymes are located mainly in the liver (22), this deactivation is suggested to play an important role in the detoxication and excretion of CP metabolites from the extracellular compartment. It seems doubtful that it can also play a significant role in the oncostatic specificity of CP as proposed by Sladek (5) and Connors et al (6) (*see* Discussion). Therefore, a new type of reaction that can also lead to deactivation of 4-hydroxy-CP under in vivo conditions has been studied intensively by our group. Since the details are discussed in other papers presented at this meeting (7,9), only some characteristics of this reaction, which might be of importance for the understanding of the problem of oncostatic specificity of CP, will be summarized:

a. 4-Hydroxy-CP reacts with thiols to yield 4-(*S-R*)-mercapto CP derivatives (9,10).

b. The rate of this reaction is very high as compared to the toxication rate of 4-hydroxy-CP. Thus, under physiologic conditions of pH and temperature the thiol reaction can proceed before alkylations occur (9).

c. By this thiol reaction 4-hydroxy-CP can be deactivated in the sense of a delayed toxication (7,9).

d. Thiols in excess over 4-hydroxy-CP completely prevent the toxication of 4-hydroxy-CP due to an equilibrium between 4-hydroxy-CP, free thiols, and the reaction product (9,10).

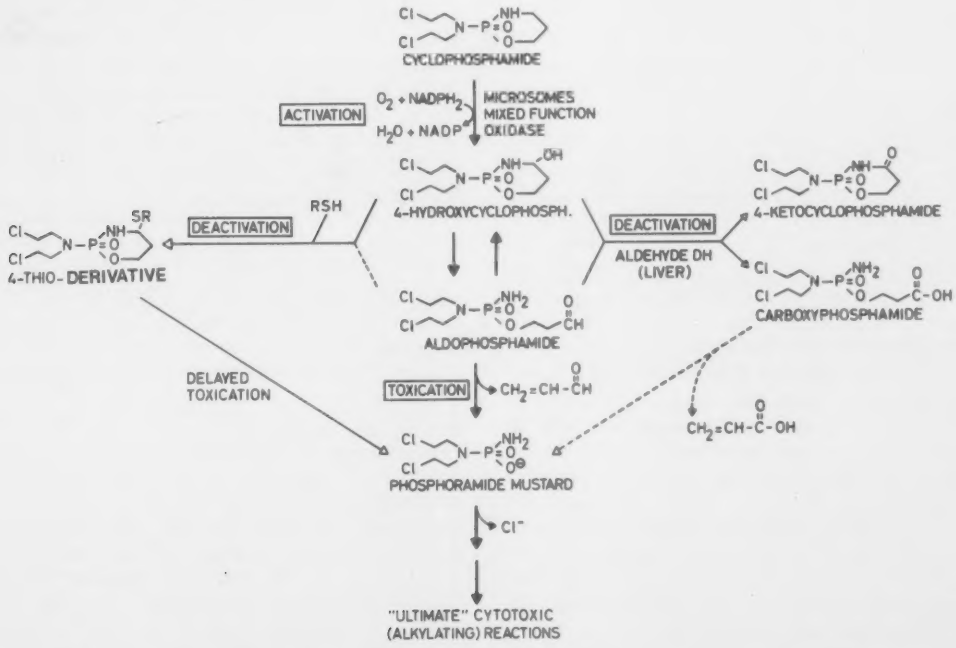


FIGURE 4.—Routes of metabolic activation and deactivation of CP are shown with regard to the reactions which either have been identified under in vivo conditions or can be postulated from in vitro data (*see* text).

e. Free protein sulfhydryl groups reacting in the same manner with 4-hydroxy-CP lead to fixation of the activated CP metabolite to the macromolecule (7).

Since the thiol reaction is peculiar to 4-hydroxy-CP these findings must be discussed with respect to their relevance for the cytotoxic specificity of 4-hydroxy-CP in vitro as well as the oncostatic specificity of CP in vivo.

DISCUSSION

The two parameters which govern the oncostatic specificity of 4-hydroxy-CP have been characterized by us in this paper and in another communication presented at this meeting by Brock (16). The cytotoxic specificity in vitro designates the molar cytotoxicity of a compound against tumor cells in suspension, related to the alkylating activity of the substance under physiologic conditions of pH and temperature (*see also* Materials and Methods), whereas the term "in vivo specificity" is defined as the ratio between lethal and curative doses, ie, the TI. From a formal point of view both kinds of specificities may well deviate from each other when determined for different compounds. This seems to be rather unlikely if one considers the basic mechanism by which the cytotoxic effect is produced, namely by alkylations. However, since both specificities exhibit high values, exceeding by one or two orders of magnitude those of the other nitrogen mustards, as has been demonstrated for 4-hydroxy-CP (table 1), there may be a direct relation between the high specific cytotoxicity in vitro and the high TI in vivo. Consequently, it is possible that the specificity of CP in vivo, which apparently is due to 4-hydroxy-CP, can also be better understood if the mechanism of cytotoxic specificity of the primary activated metabolite in vitro can be explained.

Independent of these considerations it should be stressed that any hypothesis to explain the specificity of CP action must also explain the in vitro specificity of 4-hydroxy-CP. Obviously none of the current (5,6) or previous hypotheses on CP specificity meet this task. Thus, for example, the enzymatic deactivation of the primary activated CP metabolites yielding 4-keto-CP and carboxyphosphamide may be of considerable significance for the detoxication and excretion from the extracellular compartment. However, this deactivation appears to contribute very little to the CP specificity in vivo, being apparently unable to explain this specificity of 4-hydroxy-CP in vitro. Therefore, new efforts must be made to solve this problem.

If one concentrates first on the high cytotoxic specificity of 4-hydroxy-CP in vitro (fig 1 and table 1), the question arises as to how the apparent increase of the cytotoxic effect of 4-hydroxy-CP, which exceeds the effect due to alkylation alone, can be interpreted in terms of the known metabolic pathways and reactivities of the metabolites involved. Obviously, acrolein released from aldophosphamide during toxication would be the most likely candidate for this increase of the cytotoxic effect, because of its high chemical reactivity and known toxicity in vivo. However, the apparent low molar cytotoxicity of this compound against Yoshida ascites tumor cells in vitro, as shown by Brock (16), does not seem to support this possibility. Of course, the possibility that acrolein, generated from 4-hydroxy-CP in the cell, could exert a more pronounced cytotoxic effect cannot be ruled out. Moreover, other arguments such as the dissimilarity between the toxicities of CP and acrolein with regard to their clinical phenomena, as well as the lack of cross resistance between CP and acrolein unlike other alkylating nitrogen mustards (23), do not favor a major action of acrolein as a cytotoxic component of CP. Thus, the role of acrolein during CP metabolism is most probably restricted to meeting the energy requirements for the toxication reaction as mentioned before.

Secondly, the significance of the reaction between 4-hydroxy-CP and thiols, especially free sulfhydryl groups of protein, has to be discussed in this context. This is due not only to its peculiarity to 4-hydroxy-CP, but also to its modulating effect on the toxication reaction (9) and its possible binding effect of 4-hydroxy-CP to proteins (7), including enzymes.

The thiol reaction of 4-hydroxy-CP has been intensively studied in our laboratory, as will be dealt with in other communications in this issue (7-9). Only the time course of 4-hydroxy-CP reactions under the conditions of the in vitro assay with Yoshida ascites tumor cells, including the possible role of the thiol reactions, will be discussed here. If one compares the events that may take place after incubation of micromolar concentrations of nitrogen mustard with Yoshida ascites tumor cells with those that probably occur after incubation with micromolar concentrations of 4-hydroxy-CP, one must conclude from the fast nitrogen mustard alkylation kinetics (16) that within the 2 hours after incubation all the nitrogen mustard should have alkylated nucleophilic centers in the cell, including water. During the same time, only a little 4-hydroxy-CP could have performed alkylations because of its long half-life of toxication under these conditions as shown in figure 2. On the other hand,

equimolar concentrations of both compounds exert practically the same cytotoxic effects as shown by Brock (16). Since free 4-hydroxy-CP (not having reacted by alkylations) can be washed out very easily from the tumor cells (8) during the final washing procedure before injection of the cells into rats, 4-hydroxy-CP must have been fixed in the cell by some reaction other than alkylation. It seems to be likely that the thiol reaction between 4-hydroxy-CP and free protein sulfhydryl groups is involved in that fixation due to its fast reaction kinetics when compared with the alkylation rate of 4-hydroxy-CP (7). This may lead either to a binding of the primary 4-hydroxy-CP metabolite to more sensitive (enzyme) sites in the cell or to delayed alkylations overlapping longer phases of the cell cycle of proliferating cells. In any case, by that means an increased cytotoxic efficiency of the alkylation should result, leading to the increased cytotoxic specificity as shown in table 1. If this fixation of 4-hydroxy-CP in cells prior to alkylation can be demonstrated, it must have considerable importance for the understanding of CP specificity. Indeed, first washing-out experiments with radioactive 4-hydroperoxy-CP have shown a significant retention of the activated CP in ascites tumor cells within 15 minutes at 37°C (8) when measurable alkylation is negligible. Further studies on this aspect are in progress in our laboratory.

REFERENCES

1. Brock N. Pharmacological characterization of cyclic nitrogen mustard phosphamide esters as cancer therapeutic agents. Arzneim Forsch 8:1-9, 1958.
2. Brock N, and Wilmanns H. The action of a cyclic phosphamide ester of nitrogen mustard on experimental tumors in rats: therapeutic effects and pharmacological properties of B518 ASTA. Dtsch Med Wochenschr 83:453-458, 1958.
3. Arnold H, and Bourseaux F. Synthese und Abbau cytostatisch wirksamer zyklischer *N*-Phosphamid-ester des Bis (β-chloräthyl)-amins. Angew Chem 70:539-544, 1958.
4. Brock N, and Hohorst H-J. Activation of cyclophosphamide in vivo and in vitro. Arzneim Forsch 13:1021-1031, 1963.
5. Sladek NE. Bioassay and relative cytotoxic potency of cyclophosphamide metabolites generated in vitro and in vivo. Cancer Res 33:1150-1158, 1973.
6. Connors TA, Cox PJ, Farmer PB, et al. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
7. Voelcker G, Wagner T, and Hohorst H-J. Identification and pharmacokinetics of cyclophosphamide (NSC-26271) metabolites in vivo. Cancer Treat Rep 60:415-422, 1976.
8. Draeger U, and Hohorst H-J. Permeation of cyclophosphamide (NSC-26271) metabolites into tumor cells. Cancer Treat Rep 60:423-428, 1976.
9. Draeger U, Peter G, and Hohorst H-J. Deactivation of cyclophosphamide (NSC-26271) metabolites by sulfhydryl compounds. Cancer Treat Rep 60:355-360, 1976.
10. Peter G, Wagner TH, and Hohorst H-J. Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. Cancer Treat Rep 60:429-436, 1976.
11. Takamizawa A, Matsumoto S, Iwata T, et al. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
12. Voelcker G, Draeger U, Peter G, et al. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
13. Friedman OM, and Boger E. Colorimetric estimation of nitrogen mustards in aqueous media. Anal Chem 33:906-910, 1961.
14. Schmähl D, and Druckrey H. Contributions to the mode of action of *N*-oxide of nitrogen mustard. Naturwissenschaften 43:199-203, 1956.
15. Brock N, and Schneider B. Pharmakologische Charakterisierung von Arzneimitteln mit Hilfe des therapeutischen Index. Arzneim Forsch 11:1-7, 1961.
16. Brock N. Comparative pharmacological study in vitro and in vivo with cyclophosphamide (NSC-26271), cyclophosphamide metabolites, and plain nitrogen mustard compounds. Cancer Treat Rep 60:301-308, 1976.
17. Colvin M, Padgett CA, and Fenselau C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
18. Struck RF, Kirk MC, Witt MH, et al. Isolation and mass spectral identification of blood metabolites of cyclophosphamide: evidence for phosphoramide mustard as the biologically active metabolite. Biomed Mass Spectrom 2:46-52, 1975.
19. Hohorst H-J, Ziemann A, and Brock N. Alkylierende Substanzen in Serum und Urin nach Injektion von Cyclophosphamid. Arzneim Forsch 15:432-434, 1965.
20. Hohorst H-J, Ziemann A, and Brock N. 4-Ketocyclophosphamide, a metabolite of cyclophosphamide. Formation, chemical and biological properties. Arzneim Forsch 21:1254-1257, 1971.
21. Alarcon RA, and Meienhofer J. Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide. Nature [New Biol] 233:250-252, 1971.
22. Deitrich RA. Tissue and subcellular distribution of mammalian aldehyde-oxidizing capacity. Biochem Pharmacol 15:1911-1922, 1966.
23. Lane M, and Yancey ST. Development of a leukaemia resistant to cyclophosphamide ("Cytoxan"). Nature 188:756-757, 1960.

Aldophosphamide: Synthesis, Characterization, and Comparison With "Hohorst's Aldophosphamide"[1]

Robert F. Struck[2,*]

SUMMARY

Synthesis of aldophosphamide was attempted by many standard aldehyde-forming reactions under a variety of conditions. Trace amounts of aldophosphamide were produced in several of the reactions, as judged by mass-spectral evidence. A stabilized derivative, aldophosphamide semicarbazone, was prepared and characterized by infrared and proton magnetic resonance spectroscopy. The physical characterization (infrared, proton magnetic resonance, and mass spectral analysis) of "Hohorst's aldophosphamide" failed to reveal any evidence for the existence of an aldehyde moiety, and thin-layer chromatographic comparison with the synthetic analogs diethylaldophosphamide and diethylhomoaldophosphamide indicated radical differences in migration rates between those of the analogs and that of "Hohorst's aldophosphamide." It is suggested that "Hohorst's aldophosphamide" is perhaps a diastereoisomer of 4-hydroxycyclophosphamide or an aldehyde hydrate or hemiacetal with 1,3-propanediol.

[Cancer Treat Rep 60:317-319, 1976]

In 1969, Norpoth (1) proposed that cyclophosphamide (CP) (NSC-26271; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate), after initial metabolic conversion to 4-hydroxycyclophosphamide (4-hydroxy-CP) (NSC-196562; 2*H*-1,3,2-oxazaphosphorine-4-ol, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide) yields aldophosphamide (phosphorodiamidic acid, *N,N*-bis-(2-chloroethyl)-, 3-oxopropyl ester), which subsequently yields phosphoramide mustard (NSC-69945; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-) and carboxyphosphamide (NSC-145124; hydracrylic acid, *N,N*-bis(2-chloroethyl)-phosphorodiamidate). In 1971, we proposed the possibility of a tautomeric equilibrium between 4-hydroxy-CP and aldophosphamide (2). Since evaluation of 4-ketocyclophosphamide (4-keto-CP) (NSC-139488; 2*H*-1,3,2-oxazaphosphorin-4(3*H*)-one, 2-[bis(2-chloroethyl)amino]dihydro-, 2-oxide) and carboxyphosphamide against L1210 leukemia in vivo indicated no antitumor effect for these two metabolites and since chemotherapeutic and biochemical evidence (3) implicated a metabolite intermediate between CP and carboxyphosphamide as the cytotoxic metabolite, synthesis of aldophosphamide and 4-hydroxy-CP was investigated.

Synthesis of aldophosphamide was attempted by many standard aldehyde-forming reactions (4), as summarized in table 1.

Most of these reactions yielded no detectable amount of aldophosphamide as indicated by the absence of mass-spectral peaks of m/e 227 (one chlorine, M-CH_2Cl) and m/e 136 (no chlorine, M-$N(CH_2CH_2Cl_2)$. Trace amounts of aldophosphamide were detected, as judged by mass-spectral analysis, in reaction products from aldophosphamide diethyl acetal (NSC-145932; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-, 3,3-diethoxypropyl ester), alcophosphamide (NSC-153182; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-, 3-hydroxypropyl ester), 2,3-epoxypropyl *N,N*-bis(2-chloroethyl)-phosphorodiamidate (NSC-150025), and 2-cyanoethyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (NSC-154035) (table 1; reactions 9, 17, 27, and 32). Infrared (IR) spectral analysis of these four products detected weak aldehyde carbonyl absorption, but proton magnetic resonance (PMR) spectral analysis at room temperature failed to yield a typical aldehyde proton signal at approximately 9.8 ppm, indicating either decomposition or a concentration of aldophosphamide too low to be detected.

[1]Supported by contract N01-CM-43762 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala.

*Reprint requests to: Dr. Robert F. Struck, Southern Research Institute, 2000 9th Ave S, Birmingham, Ala 35205.

TABLE 1.—Reactions investigated for the synthesis of aldophosphamide $-N2 = -N(CH_2CH_2Cl)_2$

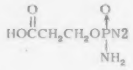
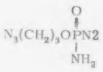
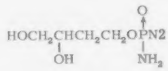
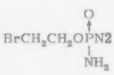
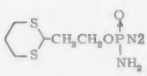
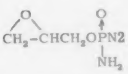
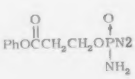
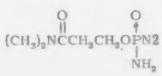
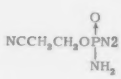
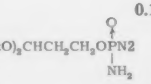
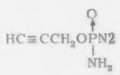
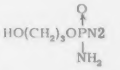
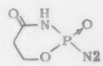
	Starting material	Reagent
1.		Diisobutylaluminum hydride
2.	(same as 1)	Lithium diethylamide
3.		Ultraviolet light irradiation
4.		Ozone, dimethyl sulfide
5.	(same as 4)	Osmium tetroxide, sodium periodate
6.		Periodic acid
7.		Disodium iron tetracarbonyl
8.		Mercuric oxide, boron trifluoride
9.		Boron trifluoride etherate
10.	(same as 9)	Aluminum trichloride
11.	(same as 9)	Hydrogen, nickel catalyst
12.	(same as 9)	*n*-Propyl iodide, sodium iodide
13.		Lithium tri-*t*-butoxy aluminum hydride
14.		Lithium triethoxy aluminum hydride
15.		Sodium aluminum hydride
16.	(same as 15)	Lithium triethoxy aluminum hydride
17.	(same as 15)	Sodium dihydrogen phosphate, nickel, pyridine
18.		0.1 N hydrochloric acid, ethanol
19.	(same as 18)	0.2 N hydrochloric acid, methanol
20.	(same as 18)	Carboxylic acid ion-exchange resin
21.	(same as 18)	Sulfonic acid ion-exchange resin
22.	(same as 18)	Acetic acid

TABLE 1.—Reactions investigated for the synthesis of aldophosphamide $-N2 = -N(CH_2CH_2Cl)_2$—Continued

	Starting material	Reagent
23.	(same as 18)	Trifluoroacetic acid
24.	(same as 18)	Chloral, carboxylic acid ion-exchange resin
25.	(same as 18)	Benzaldehyde, acetic acid
26.	(same as 18)	Ammonium chloride
27.	(same as 18)	Silica gel
28.		Diisoamylborane, hydrogen peroxide, alkali
29.		Dimethyl sulfoxide, dicyclohexylcarbodiimide
30.	(same as 29)	Dimethyl sulfoxide, oxygen
31.	(same as 29)	Dimethyl sulfoxide, argentic picolinate
32.	(same as 29)	*N*-chlorosuccinimide, dimethyl sulfide
33.		Lithium aluminum hydride

A report by Sladek (5) suggested that aldophosphamide might be stabilized in the form of its semicarbazone. Because of the difficulties in synthesizing aldophosphamide, 4-hydroxy-CP was used as a potentially latent form and did, indeed, yield aldophosphamide semicarbazone upon reaction with semicarbazide (6). Stability of the derivative was such that isolation and characterization under normal conditions were possible.

Voelcker et al (7) recently reported thin-layer chromatographic (TLC) and chemical evidence in support of the thesis that 4-hydroxy-CP and aldophosphamide exist in tautomeric equilibrium and that their respective half-lives are 20 and 80 minutes. Because of the extreme difficulties encountered in the synthesis of aldophosphamide as outlined above and as also observed by Takamizawa et al (8), the report that aldophosphamide was more stable than 4-hydroxy-CP was surprising. An attempt was made, therefore, to duplicate the results of Voelcker et al (7). Since duplication was obtained with no difficulty, more complete physical characterization of the "Hohorst's aldophosphamide" that is reported to be in equilibrium with 4-hydroxy-CP was attempted. The reported chemical and physical data (7) are not explicit for aldophosphamide (8-10).

PMR studies on the equilibrium mixture in pH 7 phosphate buffer, after TLC in ethyl acetate on

1,3-propanediol-impregnated cellulose at 5°C indicated the presence of 4-hydroxy-CP and "Hohorst's aldophosphamide" in the approximate proportions reported (7), failed to show evidence of an aldehyde proton. Also, PMR, IR, and mass spectral analysis of a chloroform extract of the equilibrium mixture gave no evidence for aldehyde, although TLC analysis indicated the presence of both components.

TLC comparison of "Hohorst's aldophosphamide" with synthetic diethylaldophosphamide (phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-*N'*,*N'*-diethyl-, 3-oxopropyl ester) and diethylhomoaldophosphamide (NSC-173967; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-*N'*,*N'*-diethyl-, 4-oxobutyl ester) (6) in the system described by Voelcker et al (7) indicated much greater mobility for both synthetic aldehydes. In fact, the R_f of both synthetic aldehydes is also greater than the R_f of CP in this system as well as in acetone:chloroform (3:1) on silica gel at −20°C.

Because of these results, it is suggested that "Hohorst's aldophosphamide" is perhaps a diastereoisomer of 4-hydroxy-CP, the isomers possibly being in equilibrium in phosphate buffer in the cold. Other 4-substituted diastereoisomers of CP have been observed (11-13). Alternatively, the unknown product may also be a hemiacetal of aldophosphamide with propanediol or an aldehyde hydrate, either of which could, upon elution and concentration in phosphate buffer in the cold, possibly regenerate 4-hydroxy-CP via aldophosphamide. Subsequent TLC analysis would then reveal regenerated 4-hydroxy-CP and the unknown product, some of which could have endured the isolation procedure or have been reformed from regenerated 4-hydroxy-CP during elution and concentration. Formation of the unknown product during the second TLC analysis appears less likely because of the failure of diethylaldophosphamide or diethylhomoaldophosphamide to yield any evidence of a related product in the same TLC system.

Takamizawa et al (8) have reported attempts to synthesize aldophosphamide from 4-keto-CP and a hemiacetal hydroperoxide, and, in the latter case, they reported IR and PMR data consistent with an aliphatic aldehyde. Reaction with dinitrophenylhydrazine, however, yielded no aldophosphamide dinitrophenylhydrazone, although IR and PMR aldehyde signals were reportedly unchanged for 15 minutes at room temperature. Data reported for *N*-3-hydroxypropylhomoaldophosphamide (phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-*N'*-3-hydroxypropyl-, 4-oxobutyl ester) (IR, 1722 cm^{-1}; PMR, δ 9.97 ppm, triplet) (8) are somewhat different from those of diethylhomoaldophosphamide (IR, 1715 cm^{-1}; PMR, δ 9.78-9.86 ppm, multiplet), which, along with diethylaldophosphamide, has also been characterized by mass spectrometry (6).

In conclusion, because of the failure of many attempts under a variety of experimental conditions, many of which were mild, by several laboratories (4,8) to produce any purified specimens of aldophosphamide, it is suggested that aldophosphamide is much less stable than 4-hydroxy-CP and that no equilibrium exists between 4-hydroxy-CP and aldophosphamide at room temperature or above.

REFERENCES

1. Norpoth K. Untersuchungen zur oxydativen umsetzung von Endoxan in vivo und in vitro. Münster, Habilitationsschrift, Fach Physiologische Chemie, Westfälischen Wilhelms-Universität, 1969.
2. Struck RF, Kirk MC, Mellett LB, et al. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
3. Hill DL, Laster WR, Jr, and Struck RF. Enzymatic metabolism of cyclophosphamide and nicotine and production of a toxic cyclophosphamide metabolite. Cancer Res 32:658-665, 1972.
4. Struck RF, and Hill DL. Investigation of the synthesis of aldophosphamide (AP), a toxic metabolite of cyclophosphamide (CTX). Proc Am Assoc Cancer Res 13:50, 1972.
5. Sladek NE. Evidence for an aldehyde possessing alkylating activity as the primary metabolite of cyclophosphamide. Cancer Res 33:651-658, 1973.
6. Struck RF. Isolation and identification of a stabilized derivative of aldophosphamide, a major metabolite of cyclophosphamide. Cancer Res 34:2933-2935, 1974.
7. Voelcker G, Draeger U, Peter G, et al. Studien zum spontanzerfall von 4-hydroxycyclophosphamid und 4-hydroperoxycyclophosphamid mit hilfe der dünnschichtchromatographie. Arzneim Forsch 24:1172-1176, 1974.
8. Takamizawa A, Matsumoto S, Iwata T, et al. Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and related compounds. J Med Chem 18:376-383, 1975.
9. Struck RF, Thorpe MC, Coburn WC, Jr, et al. Cyclophosphamide. Complete inhibition of murine leukemia L1210 in vivo by a Fenton oxidation product. J Am Chem Soc 96:313-315, 1974.
10. van der Steen J, Timmer EC, Westra JG, et al. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
11. Connors TA, Cox PJ, Farmer PB, et al. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
12. Feil VJ, and Lamoureux CJH. Alopecia activity of cyclophosphamide metabolites and related compounds in sheep. Cancer Res 34:2596-2598, 1974.
13. Cox PJ, Farmer PB, and Jarman M. The microsomal metabolites of some analogues of cyclophosphamide: 4-methylcyclophosphamide and 6-methylcyclophosphamide. Biochem Pharmacol 24:599-606, 1975.

Studies on the Selective Action of Cyclophosphamide (NSC-26271): Inactivation of the Hydroxylated Metabolite by Tissue-Soluble Enzymes[1,2]

Peter J. Cox,* Barry J. Phillips, and Peter Thomas[3,4]

SUMMARY

The hypothesis that the selective action of cyclophosphamide, compared to other nitrogen mustards, is due to a balance between enzymatic formation of inactive metabolites and chemical formation of the alkylating product was studied in view of previous observations in our laboratory. Metabolite analysis, inhibition of growth of tumor cells in culture, and kinetic analysis of relevant enzyme activities were used in this investigation. The effect of tissue-soluble enzyme fractions on biochemically prepared aldophosphamide, aldophosphamide analogs, and phosphoramide mustard showed: (*a*) a range of deactivation abilities with aldophosphamide (liver >> kidney >> intestinal mucosa > tumor > spleen = bovine serum albumin solution); (*b*) the formation of different amounts of carboxyphosphamide from aldophosphamide; and (*c*) only comparatively small reductions in the toxicity of phosphoramide mustard and of 4-hydroxy-4-methylcyclophosphamide. Correlations were found between NAD^+-dependent aldehyde dehydrogenase activity and the deactivation ability of tissue-soluble enzyme fractions. Blockage (by C4 substitution) or inhibition (by disulfiram) of secondary oxidation of aldophosphamide, mediated by aldehyde dehydrogenase, resulted in diminished deactivation ability in vitro and reduced selectivity in vivo.

[Cancer Treat Rep 60:321-326, 1976]

The metabolism of cyclophosphamide (CP) (*I*) is now reasonably well understood. Figure 1 shows the central core of the metabolic scheme containing the key reactions for antitumor activity. The first step involving the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-dependent, cytochrome P-450-linked microsomal enzyme system (1) results in 4-hydroxycyclophosphamide (4-hydroxy-CP) (*V*). This has been isolated from in vitro incubations and stabilized as a pair of diastereoisomeric ethyl derivatives (*XIX*) (2), and was first reported in 1973 (3). The hydroxy metabolite (*V*) is believed to exist both as the ring-closed form and as the ring-opened aldophosphamide (fig 1). Aldophosphamide has been positively characterized as its semicarbazone (4), although the identification of underivatized aldophosphamide formed from synthetic 4-hydroxy-CP in good yield has been claimed (5). In this paper, the terms aldophosphamide and 4-hydroxy-CP do not necessarily indicate the precise structural form or the position of the equilibrium of the two forms of the hydroxylated metabolite. Phosphoramide mustard (*IX*) has been isolated in vitro (2,6) and in vivo (7,8), and is probably derived by chemical β elimination of acrolein (*X*) from aldophosphamide, although an enzymatic mechanism cannot be entirely excluded. The relatively nontoxic metabolites excreted in the urine, carboxyphosphamide (*XVI*) and 4-ketocyclophosphamide (4-keto-CP) (*XIII*), were among the first metabolites to be identified (9,10)

[1]Supported by grant G973/787/K from the Medical Research Council, and by fellowships from Ward Blenkinsop Ltd (*Dr. Cox*), the Ludwig Institute for Cancer Research (*Dr. Phillips*), and the Alexander Keiller Fdn (*Dr. Thomas*).

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England.

[4]We wish to acknowledge Dr. M. Jarman and Mr. M. H. Baker for mass spectra determination and Mr. M. Jones for the in vivo antitumor testing results.

*Reprint requests to: Dr. Peter J. Cox, Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Rd, London SW3 6JB, England.

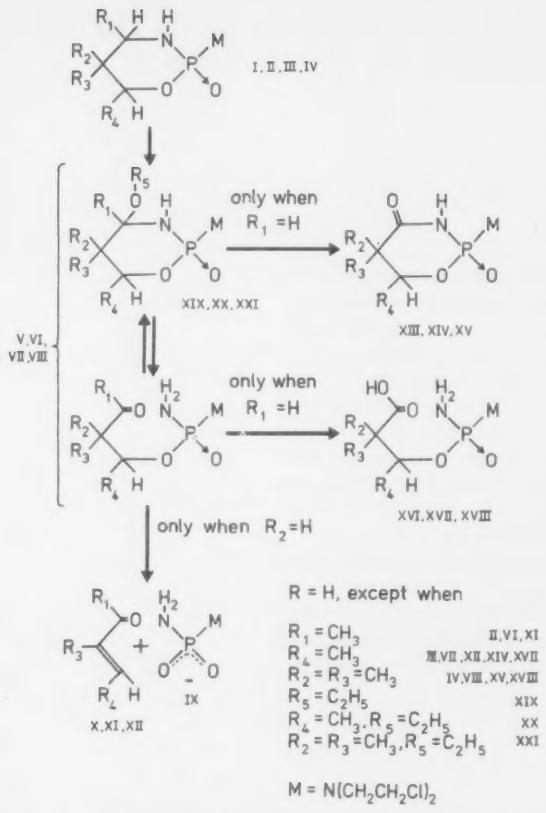

I, II, III, IV
only when R1 = H
XIX, XX, XXI
XIII, XIV, XV
V, VI, VII, VIII
only when R1 = H
XVI, XVII, XVIII
only when R2 = H
IX
X, XI, XII
R = H, except when
R1 = CH3 II, VI, XI
R4 = CH3 III, VII, XII, XIV, XVII
R2 = R3 = CH3 IV, VIII, XV, XVIII
R5 = C2H5 XIX
R4 = CH3, R5 = C2H5 XX
R2 = R3 = CH3, R5 = C2H5 XXI
M = N(CH2CH2Cl)2

FIGURE 1.—General metabolic scheme for CP and some methylated analogs.

and conversion of aldophosphamide to carboxyphosphamide by soluble enzymes in the liver has been shown (11,12). The primary metabolite (*V*) has been presumed to exert the cytotoxic effects of CP in vivo (11), although release of phosphoramide mustard has been suggested as a prerequisite for cytotoxicity (2,13). Indeed, 4-hydroxylation of 5,5-dimethylcyclophosphamide (5,5-dimethyl-CP) (*IV*) has been demonstrated (13) and the product has been shown to have very low toxicity to both normal and tumor tissue (13,14). The acyclic tautomer of *VIII* cannot release phosphoramide mustard by β elimination, which is prevented by the gem-dimethyl group at C5.

The selective action of CP in vivo, ie, its large therapeutic index (LD50/ED90), compared to those of other nitrogen mustards has been attributed (2,15) to the balance between the chemical release of phosphoramide mustard and the enzymatic formation of nontoxic products in each tissue.

MATERIALS AND METHODS

CP (*I*), 4-methylcyclophosphamide (4-methyl-CP) (*II*), and 6-methylcyclophosphamide (6-methyl-CP) (*III*) were gifts from W. B. Pharmaceuticals Ltd, Bracknell, Berkshire, England; 5,5-dimethyl-CP (*IV*) was a gift from Professor N. Brock, Asta-Werke, Bielefeld, Germany, and phosphoramide mustard (*IX*) cyclohexylammonium salt was obtained through Dr. H. Wood, National Cancer Institute, Bethesda, Md. 4-Hydroxy-5,5-dimethylcyclophosphamide (4-hydroxy-5,5-dimethyl-CP) (*VIII*) was prepared by permanganate oxidation of 5,5-dimethyl-CP (*IV*) (13), and other compounds were prepared in this institute. Acrolein was redistilled before use.

All animals were pretreated with sodium phenobarbital (1 g/liter in the drinking water) for 10 days. Disulfiram (Antabuse; bis(diethylthiocarbamyl)disulfide) was force-fed in arachis oil 16 hours prior to death at dose levels up to 400 mg/kg.

Rat tissue-soluble enzyme fractions were prepared in 0.1 M tris-HCl, pH 7.4, by standard techniques, exact details of which have been given elsewhere (16). Disruption of cells in spleen homogenate and in suspensions of bone-marrow cells, gastrointestinal mucosa cells, and Walker ascites tumor cells necessitated sonication for 30-45 seconds in an ice-bath. When necessary, tissue supernatants were dialyzed against 0.1 M tris-HCl, pH 7.4 (100 vols), for 18 hours at 4°C. Protein was measured by the method of Lowry et al (17).

Metabolism of CP by washed microsomes, which confines the enzymatic metabolism to the initial hydroxylation step, was carried out as previously described (2,16). After activation, tissue-soluble enzyme fractions (either 0.5 or 2.0 vols) were added, together with additional nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$) and nicotinamide adenine dinucleotide, oxidized form (NAD^+) (1 mg each/4.5-ml incubation), and the incubation was continued at 37°C.

The bioassay procedure and the cell-culture cytotoxicity assay were identical to those described earlier (16). For measurements of the inhibition of cell growth, an incubation of 72 hours, after a 1-hour treatment of the cells with the test material, was taken as a standard.

Formation of nicotinamide adenine dinucleotide, reduced form (NADH) was used to make kinetic measurements of tissue supernatant enzymes, alcohol and aldehyde dehydrogenases; the assays were performed at pH 7.2 in 0.1 M sodium phosphate buffer (16).

RESULTS AND DISCUSSION

Table 1 shows the toxicity to the Walker ascites tumor cells of various metabolites of CP measured by bioassay and cell-culture techniques. "Fast" and "slow" ethoxycyclophosphamide (ethoxy-CP) refer to the thin-layer chromatogram (TLC) mobility of the isomers (2). Although there are discrepancies between the results from the two techniques, which are probably due to practical differences such as the periods of treatment of the cells (bioassay 2 hrs, cell culture 1 hr), the values show general agreement that monodechloroethylation and secondary oxidation of 4-hydroxy-CP and aldophosphamide yield products with low toxicity whereas phosphoramide mustard (*IX*) is the most toxic product; compounds releasing phosphoramide mustard (ie, the ethoxy-CPs, *XIX*) show similar toxicity to activated CP prepared by microsomal incubation. The toxicities of various metabolites in a metabolism mixture have been shown using a technique whereby a TLC was scanned for radioactivity and for cytotoxicity (18), the results of which agree with the measurements made on isolated materials.

CP, activated at 75 μg/ml, further incubated with 0.5 volumes of tissue supernatant or buffer, and bioassayed (so that the final concentration of metabolites was 5 μg/ml), was deactivated by liver- and kidney-soluble enzymes but not by supernatants from gut mucosa, Walker ascites tumor cells, or bone marrow. Figure 2 shows representative results for incubation with liver supernatant. The presence of sufficient metabolite after 24 hours, when diluted only with buffer, to cause 99% cell kill, suggests that it is 4-hydroxy-CP (*V*) and not phosphoramide mustard (*IX*) in solution, since we have previously shown the short half-life of phosphoramide mustard under identical conditions (2). The 4-hydroxy-CP can then release *IX* inside the tumor cells. On chemical grounds, the formation of 4-hydroxy-CP does not activate the mustard side chain, whereas the negative charge of phosphoramide mustard labilizes the chlorine atoms.

TABLE 1.—Concentrations of CP and metabolites required to kill 50% of Walker ascites tumor cells in the bioassay system and in cell culture

Compounds	Concentration (μM)	
	Bioassay	Cell culture
CP (*I*)	1540	23,000
Monochloroethylcyclophosphamide	4050	>15,000
4-Keto-CP (*XIII*)	2920	880
Carboxyphosphamide (*XVI*)	> 685	1370
Acrolein (*X*)	62.5	17.9
"Slow" 4-ethoxy-CP (*XIX*)	6.6	26.3
"Fast" 4-ethoxy-CP (*XIX*)	1.45	7.2
Phosphoramide mustard (*IX*)	1.56	1.88
CP + microsomes + cofactors	5.8	2.1

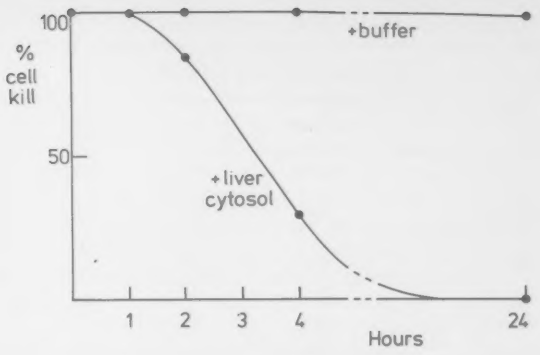


FIGURE 2.—Bioassay results for activated CP (5 μg/ml) in the presence and absence of liver-cell cytosol using Walker ascites tumor cells.

The half-lives of 4-hydroxy-CP and aldophosphamide have been reported to be 20 and 80 minutes respectively in phosphate buffer, pH 7.2 (5). Our experiments described above suggest that the half-life for activated CP is about 18 hours in tris-HCl, pH 7.4, indicating possible stabilization of 4-hydroxy-CP by the tris(hydroxymethyl)methylammonium ion. Thus, half-life measurements in buffer may have little relevance to the in vivo situation in the case of CP metabolites.

The bioassay experiments were repeated using inhibition of cell growth in culture as the cytotoxicity index. In order to compare the effects of various tissue-soluble enzyme fractions (2 volumes incubated with activated CP for 2 hrs at 37°C), dose-response curves were constructed for each supernatant and ID50 values were measured. Dose-reduction factors were then calculated compared to the ID50 of activated CP diluted only with buffer (table 2). Also shown in table 2 are dose-reduction factors for phosphoramide mustard (*IX*). The value for *IX* with liver-cell cytosol is much less than for activated CP and shows that the varied deactivation activities of the supernatants are specific processes and not merely due to the removal of alkylating functions.

Figure 3 shows dose-response curves for CP (*I*), 4-methyl-CP (*II*), and 6-methyl-CP (*III*) after activation with microsomes and subsequent incubation with either buffer or liver-cell cytosol. Dose-reduction factors calculated from these curves were 49.1

TABLE 2.—Dose-reduction factors for activated CP and for phosphoramide mustard when incubated with tissue-soluble enzymes, compared to incubation with buffer alone

Source of soluble enzymes	Protein concentration (mg/ml)	Dose-reduction factor	
		Activated CP	Phosphoramide mustard
Bovine serum albumin solution	16.0	1.4	0.95
Spleen	20.4	1.3	1.3
Tumor	15.4	2.9	—
Mucosa	12.9	7.3	—
Kidney	16.8	17.0	—
Liver	24.0	49.1	2.7

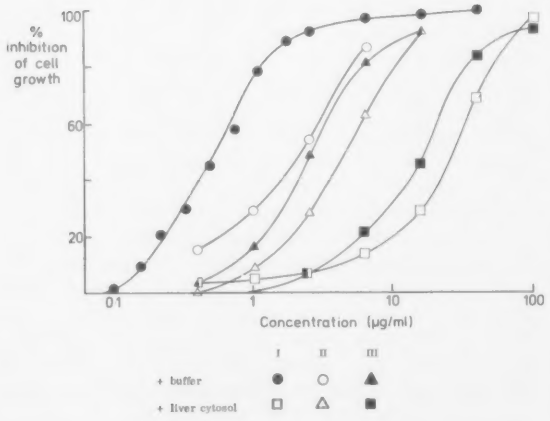


FIGURE 3.—Dose-response curves relating inhibition of Walker ascites tumor cell growth in culture to the substrate concentration. Substrate was metabolized with microsomes and cofactors prior to dilution with buffer or liver-cell cytosol, and further incubation for 2 hrs at 37°C. After centrifugation, the supernatants were tested for cytotoxicity.

(CP), 6.9 (6-methyl-CP analog), and 2.2 (4-methyl-CP analog), showing that liver-cell cytosol has no specific effect on 4-hydroxy-4-methylcyclophosphamide (4-hydroxy-4-methyl-CP). These results are in agreement with the therapeutic indices of the parent compounds in vivo (table 3). The therapeutic index of 6-methyl-CP is the same as that of CP, whereas that for 4-methyl-CP is similar to phosphoramide mustard itself. The LD50 and ED90 values for phosphoramide mustard are smaller because activation reactions are not required for cytotoxicity. The results in vivo and in vitro can be rationalized from the metabolism of the compounds (fig 1). 6-Methyl-CP can undergo all the

TABLE 3.—Screening data for the ADJ/PC6 mouse plasma cell tumor

Compounds	LD50 (mg/kg)	ED90 (mg/kg)	Therapeutic index* (LD50/ED90)
CP (*I*)	335	3.6	93
6-Methyl-CP (*III*)	340	3.7	92
4-Methyl-CP (*II*)	430	8	54
Phosphoramide mustard (*IX*)	141	2.8	50
CP + disulfiram (2 × 100 mg/kg 2 hrs before and after CP)	238	3.75	63

*Values shown cannot be demonstrated to be statistically significantly different because the number of experiments was too small. However, the trend in the values is important.

reactions described for CP. The isolation of two isomeric 4-ethoxy derivatives (*XX*) and crotonaldehyde (*XII*) as its 2,4-dinitrophenylhydrazone has been reported (15). 4-Methyl-CP, however, cannot undergo secondary oxidation after the initial hydroxylation step due to the presence of the methyl group. Methyl vinyl ketone (*XI*), again as its 2,4-dinitrophenylhydrazone, and 4-hydroxy-4-methyl-CP phosphamide (*VI*), probably as the acyclic tautomer, have been isolated (15). The absence of further oxidation was shown by permanganate oxidation of 4-methyl-CP yielding the hydroxy derivative (15), whereas permanganate oxidation of CP yields 4-keto-CP (19).

5,5-Dimethyl-CP (*IV*) shows no measurable effect on tumor growth in vivo. This is thought to be due to the prevention of release of phosphoramide mustard by the C5 gem-dimethyl grouping. Hydroxylation of *IV* has been shown and the product (*VIII*), together with an ethyl derivative (*XXI*), was isolated (13) after incubation with microsomes and cofactors. Both *VIII* and *XXI* were unreactive towards the 4-(*p*-nitrobenzyl)pyridine TLC spray reagent for alkylating agents. Aqueous permanganate oxidation of *IV* yielded both *VIII* and the keto compound (*XV*) (13).

Comparison of dose-reduction factors with the activities of appropriate soluble enzymes in the tissue supernatants (alcohol and aldehyde dehydrogenases), measured with model substrates, showed a qualitative correspondence with aldehyde dehydrogenase levels. Using 4-hydroxy-5,5-dimethyl-CP (*VIII*) as a test substrate accentuated the differences between tissues (table 4). The low activity of the tumor-cell supernatant was particularly noticeable. Reaction rates with "real" substrates were much lower than with "model" substrates

TABLE 4.—Relative rates of NADH production by tissue-soluble enzyme fractions

Source of soluble enzyme fraction	Substrate	
	Acetaldehyde	4-Hydroxy-5,5-dimethyl-CP
Liver	100	100
Kidney	108	—
Intestinal mucosa	41	27
Walker ascites tumor cell	27	8
Spleen	13	—

(table 5). As anticipated, 4-hydroxy-4-methyl-CP caused no NADH formation when incubated with liver-cell cytosol and NAD^+. This confirmed that enzymatic secondary oxidation of this compound was blocked by the methyl group.

Inhibition of aldehyde dehydrogenase in vivo with disulfiram (*see* Materials and Methods) resulted in a 10% residual acetaldehyde-metabolizing activity in liver-cell cytosol, and reduced the dose reduction factor to approximately 30% that of normal liver-cell cytosol. Thus, an ID50 of $\sim 8\,\mu g$/ml was measured for activated CP and liver-cell cytosol (disulfiram treated), which compares to an ID50 of 4.8 μg/ml of 4-hydroxy-4-methyl-CP with normal liver-cell cytosol. Disulfiram given in combination with CP in vivo reduced the latter's therapeutic index to 63 (table 3). Experiments with ^{14}C-CP (New England Nuclear, Swindon, England) have shown different extents of carboxyphosphamide formation by gastrointestinal mucosa and liver-cell cytosol under the experimental conditions described for the deactivation experiments (16). Preliminary results suggest that carboxyphosphamide formation is reduced progressively when liver supernatants from animals treated with increasing doses of disulfiram are used.

TABLE 5.—Relative rates of NADH production by liver-cell-soluble enzymes with different substrates

Substrate	Relative rate
Acetaldehyde	100
Aldophosphamide (*V*)*	~ 2.5
Analogs from:	
5,5-Dimethyl-CP (*VIII*)*	~ 1.0
6-Methyl-CP (*VII*)*	~ 0.8
4-Methyl-CP (*VI*)*	0

*Prepared biochemically by incubation of parent compound (1 mM) with washed liver microsomes and cofactors at 37°C for 45 mins (*see* Materials and Methods), conditions under which metabolism approaches 100%. Microsomes were removed by centrifugation at 65,000 g_{av} for 30 mins at 0°C, and the clear supernatants were used as substrate solutions in the enzyme assay.

As reported elsewhere in this issue (20), substitution of deuterium at the C4 position in CP resulted in an isotope effect of approximately 1.8 for the secondary oxidation of aldophosphamide to carboxyphosphamide, but did not affect the therapeutic index in vivo. Superficially, this appears to be in conflict with the work reported in this paper. However, taking the example of the effect of disulfiram pretreatment, the reduction of liver aldehyde dehydrogenase activity was equivalent to an isotope effect of 10; this resulted in a 70% reduction in the deactivation ability of liver-cell cytosol (measured by cell-culture experiments) and the loss of CP's selectivity in vivo when compared to phosphoramide mustard (table 3). In the context of this particular reaction, an in vitro deuterium isotope effect of 2 may be expected to have only a small biologic effect.

In a recent report (7) of the presence of phosphoramide mustard and not 4-hydroxy-CP in the blood of mice treated with CP, it was suggested that phosphoramide mustard, formed in the liver and released into the blood stream, is the compound causing cytotoxicity. Although there is little doubt that phosphoramide mustard is the toxic product, its release into the extracellular fluid at the liver is difficult to rationalize with the selective action of CP. In our in vivo tumor test systems, CP has twice the therapeutic index of phosphoramide mustard. Although this may be explicable on pharmacokinetic grounds, the therapeutic index of 4-methyl-CP is similar to that of phosphoramide mustard. This shows that insertion of the C4-methyl group, which blocks secondary enzymatic oxidation, into the CP structure reduces the selectivity of the compound in vivo to the level of the final alkylating product, phosphoramide mustard.

Within the limitations of the in vitro and in vivo systems used, the results we have obtained lead us to believe that enzymatic oxidation of aldophosphamide to carboxyphosphamide makes an important contribution to the selective action of CP, although participation of other mechanisms is not excluded.

REFERENCES

1. COHEN JL, and JAO JY. Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat. J Pharmacol Exp Ther 174:206-210, 1970.
2. CONNORS TA, COX PJ, FARMER PB, ET AL. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
3. Cox PJ. Evidence for the active intermediate in cyclophosphamide metabolism. Br J Cancer 28:81, 1973.

4. Struck RF. Isolation and identification of a stabilized derivative of aldophosphamide, a major metabolite of cyclophosphamide. Cancer Res 34:2933-2935, 1974.
5. Voelcker G, Draeger U, Peter G, et al. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
6. Colvin M, Padgett CA, and Fenselau C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
7. Struck RF, Kirk MC, Witt MH, et al. Isolation and mass spectral identification of blood metabolites of cyclophosphamide: evidence for phosphoramide mustard as the biologically active metabolite. Biomed Mass Spectrom 2:46-52, 1975.
8. Fenselau C, Kan M-NN, Billets S, et al. Identification of phosphorodiamidic acid mustard as a human metabolite of cyclophosphamide. Cancer Res 35:1453-1457, 1975.
9. Struck RF, Kirk MC, Mellett LB, et al. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
10. Takamizawa A, Tochino Y, Hamashima Y, et al. Studies on cyclophosphamide metabolites and their related compounds. I. Chem Pharm Bull 20:1612-1616, 1972.
11. Hill DL, Laster WR, Jr. and Struck RF. Enzymatic metabolism of cyclophosphamide and nicotine and production of a toxic cyclophosphamide metabolite. Cancer Res 32:658-665, 1972.
12. Sladek NE. Evidence for an aldehyde possessing alkylating activity as a primary metabolite of cyclophosphamide. Cancer Res 33:651-658, 1973.
13. Cox PJ, Farmer PB, and Jarman M. The use of cyclophosphamide analogues in mechanistic studies of the metabolism of cyclophosphamide. *In* Advances in Mass Spectrometry in Biochemistry and Medicine (Frigerio A, and Castagnoli N, eds). New York, Spectrum Publications. In press, 1976.
14. Arnold H, Bourseaux F, and Brock N. Relationship between chemical constitution and carcinotoxic activity of the phosphamide esters of bis(β-chloroethyl)amine. Arzneim Forsch 11:143-158, 1961.
15. Cox PJ, Farmer PB, and Jarman M. The microsomal metabolism of some analogues of cyclophosphamide: 4-methylcyclophosphamide and 6-methylcyclophosphamide. Biochem Pharmacol 24:599-606, 1975.
16. Cox PJ, Phillips BJ, and Thomas P. The enzymatic basis of the selective action of cyclophosphamide. Cancer Res 35:3755-3761, 1975.
17. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951.
18. Phillips BJ. A simple, small scale cytotoxicity test, and its uses in drug metabolism studies. Biochem Pharmacol 23:131-138, 1974.
19. Jarman M. Formation of 4-ketocyclophosphamide by the oxidation of cyclophosphamide with $KMnO_4$. Experientia 29:812-814, 1973.
20. Cox PJ, Farmer PB, Foster AB, et al. The use of deuterated analogs in qualitative and quantitative investigations of the metabolism of cyclophosphamide (NSC-26271). Cancer Treat Rep 60:483-491, 1976.

Studies on the In Vivo Formation of Acrolein: 3-Hydroxypropylmercapturic Acid as an Index of Cyclophosphamide (NSC-26271) Activation[1,2]

R. A. Alarcon[3,4,*]

SUMMARY

3-Hydroxypropylmercapturic acid (MCA) has been quantitatively determined in the urine of rats given cyclophosphamide (CP), related antineoplastic agents, allyl alcohol, or acrolein, with a simple procedure involving the use of an amino-acid analyzer. Male rats (300-400 g) injected with CP (50 mg [179.1 μmols]/kg of body weight) excreted 16.7 μmols of MCA/kg in their 24-hour urine. Equivalent amounts of isophosphamide produced 9.0; triphosphamide, 16.1; ASTA-5607, 7.2; ASTA-5122, 4.1; and cytoxyl alcohol, 0.4 μmols of MCA/kg. From allyl alcohol and acrolein, 26.3 and 19.7 μmols of MCA/kg were obtained respectively. MCA values were directly proportional to drug dose levels. Since acrolein and phosphorodiamidic acid mustard are the toxic decomposition products of aldophosphamide, and acrolein conjugation with glutathione appears to be the first step for MCA formation, values for MCA would reflect active CP levels. The in vitro interaction of acrolein with glutathione, other sulfhydryl compounds, and a few amino acids at concentrations of 0.15 μmols/ml was also studied. The decrease of acrolein's main absorption peak at 209 nm was used to follow its reaction rate. The faster interactions observed were with the sulfhydryl compounds, where a 50% decrease of absorption in interactions with glutathione and cysteine (at pH 7.4 and 23°C) took place in 111 and 30 seconds respectively. Incubation of these adducts at 37°C and 100°C generated acrolein with a maximum recovery yield of 83% at 100°C. Five patients given 1 g of CP iv excreted 6.4-50 μmols of MCA in their urine in 6 hours.

[Cancer Treat Rep 60:327-335, 1976]

The formation of acrolein from cyclophosphamide (CP) and related antineoplastic agents during their in vitro oxidation, whether by liver microsomes or by simple chemicals, was shown in our laboratories in 1971 (1) and confirmed in several others (2-5). The search for acrolein as a possible product of CP was based on the similarities existing between the structures of oxidized polyamines (unstable acrolein-generating compounds [6-8]) with the hypothetical primary metabolites advanced by Brock and Hohorst (9) and Norpoth et al (10). The finding of acrolein brought with it implications that played an important role in the elucidation of the metabolic pathway of CP. It strongly supported the existence of the hypothetical primary metabolites. It made apparent that elimination of acrolein from either of these structures, whether formed by *O*-dealkylation or *N*-dealkylation, left phosphorodiamidic acid mustard as the residual part of the molecule. This latter compound, a nitrogen mustard synthesized with several others by Friedman et al (11), was already known to be an effective antitumor agent in experimental animals (12) but rather ineffective in clinical trials (13).

[1]Supported by Public Health Service grant AM-00567 from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Department of Health, Education, and Welfare.

[2]3-Hydroxypropylmercapturic acid: CAS reg. No. 23127-40-4.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Department of Biological Chemistry, Harvard Medical School, and the Cancer Research Institute, New England Deaconess Hospital, Boston, Mass.

[4]I wish to acknowledge the support and valuable advice of Dr. W. Eugene Knox and the excellent technical assistance of Barbara Green.

*Reprint requests to: Dr. R. A. Alarcon, Cancer Research Institute, New England Deaconess Hospital, 185 Pilgrim Rd, Boston, Mass 02215.

An indirect identification of aldophosphamide was advanced by Hill et al in 1972 (14), and the conclusive identification of its semicarbazone derivative was achieved recently (15). In 1973 Colvin et al (2) and Connors et al (4) independently identified the phosphorodiamidic acid mustard as a CP degradation product in vitro, and more recently its in vivo detection was successfully accomplished (16).

In the meantime, in a series of abstracts and in a detailed paper, Kaye's group (17-20) reported the presence of 3-hydroxypropylmercapturic acid (MCA) in the urine of rats given allyl alcohol, several allyl compounds, or acrolein. This compound was isolated as a dicyclohexylammonium salt from the combined urine of a large number of rats (144 received acrolein). Acrolein appeared to be the required intermediate, formed in vivo from the allyl compounds, that conjugates with glutathione to initiate the metabolic chain of reactions leading to the MCA formation. Gray and Barnsley (21) proposed a similar suggestion to explain the resulting MCA after administration to experimental animals of crotyl compounds or crotonaldehyde. More recently, Kaye and Young (22) reported in a brief paper the qualitative detection of MCA in the urine of rats and patients given CP.

Work in our laboratories confirmed the initial finding by Kaye (20) on the presence of MCA in the urine of rats given acrolein, and we independently detected this MCA in the urine of rats given CP. The development of a procedure that could be used to quantitate this MCA in the urine of individual experimental animals or man took first priority. This was considered important and potentially valuable to pharmacologic studies on CP since MCA values were expected to be proportional to the fraction of CP metabolized through its active pathway. Neither acrolein nor MCA would be formed from the inactive metabolites of CP.

The purpose of this paper is to report this procedure and the MCA amounts excreted by individual experimental animals given CP, a series of related antineoplastic agents, allyl alcohol, and acrolein. Also, a preliminary report on the amounts of MCA excreted by a small group of patients receiving CP is included, as well as in vitro studies related to the first accepted step in MCA formation, the conjugation of acrolein with glutathione.

MATERIALS AND METHODS

Drugs.—CP was purchased from Mead and Johnson, Evansville, Ind. Isophosphamide (IP), triphosphamide, and cytoxyl alcohol were obtained from Dr. A. R. Stanley, Drug Development Branch, Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. ASTA-5607 and ASTA-5122 were made available by the courtesy of Dr. N. Brock, Pharmacology Laboratories, Asta-Werke AG, Brackwede, Germany.

Chemicals.—Acrolein, reagent grade, was purchased from Eastman Kodak Co, Rochester, NY. *S*-(3-hydroxypropyl)-L-cysteine (3-H Pr Cyst) and *S*-(2-hydroxypropyl)-L-cysteine were synthesized according to Barnsley (23). MCA, as its dicyclohexylammonium salt, was synthesized according to the procedure reported by Kaye et al (24). All other chemicals were obtained from commercial sources in the highest degree of purity available.

Acrolein-sulfhydryl compound adducts.—These adducts were prepared by mixing 15 μmols of acrolein with equivalent amounts of the sulfhydryl compound in 100 ml of 0.02 M phosphate buffer (Na_2HPO_4-KH_2PO_4) at pH values of 7, 7.4, and 8.5. The mixtures were left at room temperature (23°C-25°C) for various lengths of time (see Results).

Adduct reduction.—Two mg of sodium borohydride in 0.2 N NaOH (0.2 ml) was added to 8-ml solutions (pH 7.4) of acrolein with the sulfhydryl compound (2.5 μmols/ml) after incubation at room temperature for 25 minutes, and left for 1 hour undisturbed before starting identification procedures.

Iodometric titrations.—Glutathione and acrolein-sulfhydryl compound adducts were titrated according to Woodward and Fry (25).

Procedures for acrolein regeneration.—Two sets of experiments, at 37°C and 100°C, were carried out to study the regeneration of acrolein from the above adducts. The procedures used were essentially similar to those formerly used for oxidized CP (1). The above adducts were contained in 250-ml two-necked round-bottomed flasks either in a 37°C waterbath or on heating mantles. One of the openings was the inlet for the carrier gas (air, 130-140 ml/min) and the other (outlet) was connected to a cooled 400-mm West condenser whose upper end was closed by an adaptor connected by rubber tubing to a gas absorption tube (the cold trap). This trap, containing 150 ml of water, was surrounded by crushed ice and served to trap and accumulate any released acrolein carried by the air bubbling through it. The trapping efficiency of these cold traps for 15 μmols of acrolein in 100-ml 0.02 M phosphate buffer solutions (pH 7.4) at 100°C was 80%-90%.

Determination of acrolein.—Acrolein was determined in the cold trap solutions by fluorometry as well as by ultraviolet spectrometry as described in detail in our previous studies (6,26,27). Ultraviolet spectra were recorded in the 200-250-nm region where the main absorption peak of acrolein occurs

(209 nm). The decrease of this peak was followed as an index of the reaction rate of acrolein with the diverse adduct components studied. A Unicam SP-800 (Phillips Electronics Instruments, Mt. Vernon, NY) was used.

Experiments with animals.—Male rats (300-400 g, Kx strain, obtained from the New England Deaconess Hospital stock) were used in these studies. CP and related drugs were administered ip. Acrolein and allyl alcohol in peanut oil were given sc after light ether anesthesia. After the doses were given the rats were placed in metabolic cages and urine was collected for 24 hours in bottles surrounded by crushed ice.

Paper chromatography studies.—Ascending chromatograms using Whatmann 3 MM paper in an acidic solvent mixture (*n*-butanol:water:acetic acid, 12:5:3 by vol) and in a basic one (*n*-butanol:pyridine:3 M NH_3, 80:20:1 by vol) were developed for 8-9 hours. Concentration of the urine by methyl ethyl ketone extraction and the rest of the procedures to detect sulfur-containing compounds with chloroplatinic acid reagent (28) and amino acids with ninhydrin, followed the guidelines suggested by Kaye (20).

MCA quantitation method.—(*a*) Hydrolysis procedure: The urine from treated or control rats was filtered on paper, and the aliquots were subjected to hydrolysis in sealed ampules at a 2 N HCl molarity at 100°C for 3 hours. Authentic MCA in aqueous solutions or in normal urine was similarly processed; 0.2 or 0.4 ml of the hydrolysate added to 1.8 or 1.6 ml of 0.2 M sodium citrate buffer (pH 2.2) and the mixture injected into the amino-acid analyzer. (*b*) Amino-acid determinations: Amino-acid analyses were performed in a Beckman Amino-Acid Analyzer Model 120 C. Most of the hydrolyzed urine analyses were carried out with the acid-neutral column, using 0.2 M sodium citrate buffer, pH 3.18, following routine procedures described for physiologic fluids (29). Authentic 3-H Pr Cyst was used to prepare the standard solutions.

Patient studies.—The small number of patients receiving CP and studied thus far for the presence of MCA in their urine, were unselected with regard to concurrent treatments or type of disease.

RESULTS

Qualitative Determination of MCA

The presence of MCA in the urine of rats given acrolein was confirmed in a series of preliminary experiments following the procedures reported by Kaye (20). The 24-hour urine collected from four rats (330-350 g in weight, Kx strain) injected sc with 150 μmols of acrolein in peanut oil, was extracted with methyl ethyl ketone, and the extract was evaporated to dryness, dissolved in 2 ml of methanol, and paper chromatographed in acidic and basic solvent systems (*see* Methods). The chromatograms were stained with the chloroplatinic acid reagent, used to detect sulfur-containing compounds (28), and with ninhydrin. As expected, one white spot appeared with the chloroplatinic acid, but none with ninhydrin. The R_f values on ascending chromatograms were 0.72 in the acidic solvent and 0.40 in the basic one. Acid hydrolysis of this compound resulted in a secondary product that gave a white spot with the chloroplatinic reagent and a blue one with ninhydrin and had an R_f value of 0.23 in the first solvent and a value of 0.28 in the latter one. Subsequently, two rats (360 g) were injected with 30 mg of CP ip, and the urine extracted as above. This new extract and its acid hydrolysis product were chromatographed alongside the former compounds obtained from the acrolein-treated rats, and with authentic MCA and 3-H Pr Cyst. Identical staining characteristics and R_f values were obtained for the three pairs of compounds. Furthermore, as expected, the acid hydrolysis of the synthetic MCA resulted in the formation of 3-H Pr Cyst.

Having established qualitatively that MCA was present in the urine of rats given acrolein or CP, efforts were directed to develop a procedure to quantitate MCA in individual experimental animals. Use of an amino-acid analyzer to measure the hydrolytic product of MCA (3-H Pr Cyst) appeared feasible and the following experimental basis was established.

a. Synthetic 3-H Pr Cyst gave only one peak in the amino-acid analyzer chromatogram in the region corresponding approximately to 130 minutes of elution time using the acid-neutral column. Continuing the chromatogram with the basic column, no additional peaks were detected.

b. Synthetic MCA hydrolyzed for 3 hours in 2 N HCl in sealed ampules at 100°C resulted in the formation of equimolar amounts of 3-H Pr Cyst.

c. Synthetic MCA added to normal rat urine and similarly hydrolyzed behaved as above, and most important, no constituent from the hydrolyzed urine interfered with the 3-H Pr Cyst peak (fig 1). This peak appeared after serine (approximately 30 mins) and before glutamic acid (approximately 15 mins). Proline, a small peak 5 minutes after 3-H Pr Cyst, disappeared when higher concentrations of this latter compound were recorded.

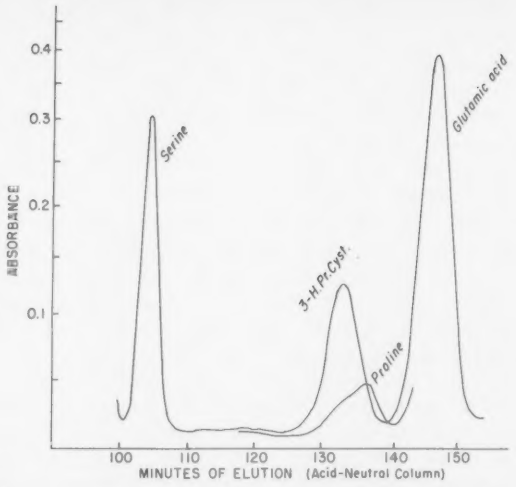


FIGURE 1.—Amino-acid analyzer chromatogram of hydrolyzed urine from a rat (355 g) given 93 μmols of CP (75 mg/kg rat body weight). MCA measured in 24-hr urine, 9.65 μmols (10.3% yield).

d. Urine from untreated rats before or after hydrolysis did not show any inflection in the chromatograms corresponding to 3-H Pr Cyst.

e. S-(2-hydroxypropyl)-L-cysteine appeared in the chromatogram approximately 40 minutes before its isomer.

Quantitation of MCA in Urine of Experimental Animals

The procedure finally devised involved acid hydrolysis and the use of the amino-acid analyzer (*see* Methods). Following this method, the amounts of MCA represented in figure 2 were obtained. MCA was measured in rat urine collected during the first 24 hours after administration of CP, isophosphamide (IP), or acrolein, at diverse dose levels. Three male rats (300-400 g) were used at each level. The dose range of acrolein was from 50 to 300 μmols/kg rat body weight. For the two drugs, a rather similar dose range was investigated: between 12.5 and 75 mg/kg body weight for CP and equivalent amounts (except for the lowest dose) for IP. The amounts of MCA excreted were directly proportional to the amounts of drug given. The average percentage yields of MCA from CP (9.7%) and IP (4.7%) remained practically constant at all dose levels. For acrolein, however, the yields varied from 17.6% for the lowest doses to 10% for the higher ones, where wider individual variation appeared.

Several related antineoplastic agents were compared for their ability to produce MCA at a dose

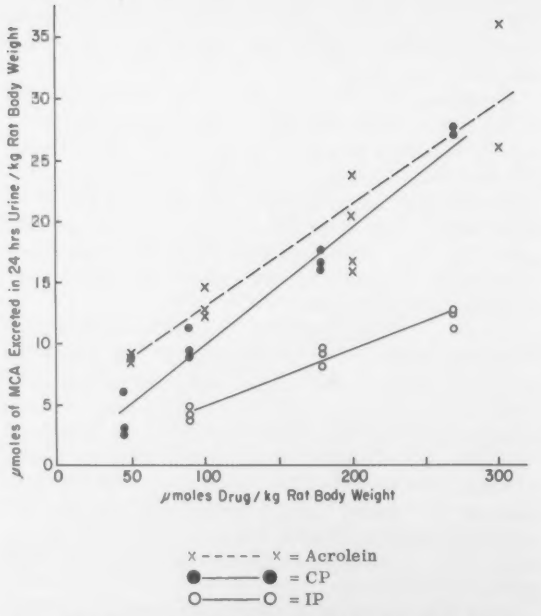


FIGURE 2.—Comparison of the amounts of MCA excreted by rats given acrolein, CP, and IP.

level of 179.1 μmols/kg rat body weight, a dose which is equivalent to 50 mg/kg of CP. Among the drugs listed in table 1, the highest yields of MCA were given by CP followed by triphosphamide; cytoxyl alcohol gave the lowest amounts of MCA. The most effective compounds in the formation of MCA studied to date, however, are allyl alcohol followed by acrolein. Also forming part of table 1 are the in vitro amounts of acrolein generated from 20 μmols of these drugs during the first 2 hours of oxidation with modified Fenton's reagent at 37°C. MCA and acrolein values (for this short period of oxidation studied) appear to correlate well only for CP and IP and to a lesser degree for cytoxyl alcohol. For the rest of the compounds, no correlations are apparent. Two rats injected with 5 and 10 μmols of MCA/rat excreted 60% and 65% respectively of this presumed end product in their 24-hour urine.

Quantitation of MCA in Human Urine

Using identical methods as described for the experimental animals, aliquots of urine were hydrolyzed and subjected to amino-acid analysis. The five patients studied were unselected and were receiving treatment with multiple drugs. The main

TABLE 1.—Comparison on the in vivo formation of MCA and in vitro formation of acrolein, from CP and related compounds

Compound injected (179.1 μmols/kg*)	MCA (μmols/kg) in first 24-hr urine	Yield (%)	Acrolein collected (μmols) from 20 μmols of compound after 2-hr oxidation†	Yield (%)
CP	16.7	9.3	2.72	13.6
IP	9.1	5.1	1.57	7.8
Triphosphamide	16.1	8.9	1.35	6.7
ASTA-5607	7.3	4.0	2.06	10.3
ASTA-5122	4.1	2.3	1.95	9.7
Cytoxyl alcohol	0.4	0.2	0.58	2.9
Acrolein	19.7	11.0		
Allyl alcohol	26.3	14.7		

*Equivalent to 50 mg of CP/kg rat body wt. Three rats were used in each experiment.

†Modified Fenton's reagent (hydrogen peroxide, 266 μmols; ferrous sulfate, 30 μmols; edetic acid, 53 μmols; 0.2 M phosphate buffer, pH 7; final vol, 10 ml), 37°C.

objective was to test the feasibility of this method to measure MCA in man. All patients received 1 g of CP iv, and their urine was collected for 6 hours.

The amounts of MCA varied widely. The lowest levels found were 6.4 and 9.9 μmols, one intermediate value was 17.5 μmols, and the highest values found were 40 and 50 μmols. If the dose of CP given was expressed in mg/kg, the dosage fluctuated between 10 and 18 mg/kg. No correlation between dose values and amounts of MCA was apparent.

In Vitro Studies on Acrolein-Sulfhydryl Compound Adducts

According to the extensive literature on MCA formation (30), the first step in the series of reactions leading to MCA would be the conjugation of acrolein with glutathione. Taking advantage of the high molecular extinction coefficient of acrolein at 209 nm (11,540), it was thought that its conjugation with glutathione, postulated to occur at the acrolein double-bond reactive site, could be followed by recording the decrease of absorbance at this wavelength as the reaction proceeded. The concentration of acrolein chosen was 0.15 μmols/ml 0.02 M phosphate buffer, whose spectrum in the Unicam SP-800 with an absorbance scale up to 2.0, gave an absorbance maximum around 1.72. A similar concentration of glutathione gave at this wavelength an absorbance value of only 0.6. Mixing equimolar amounts of both compounds and incubating the mixture at room temperature (23°C), the expected decrease took place in a short period of time (fig 3). In table 2 the time periods are recorded in which a 50% decrease occurred when acrolein interacted with diverse sulfhydryl compounds at room temperature and at three different

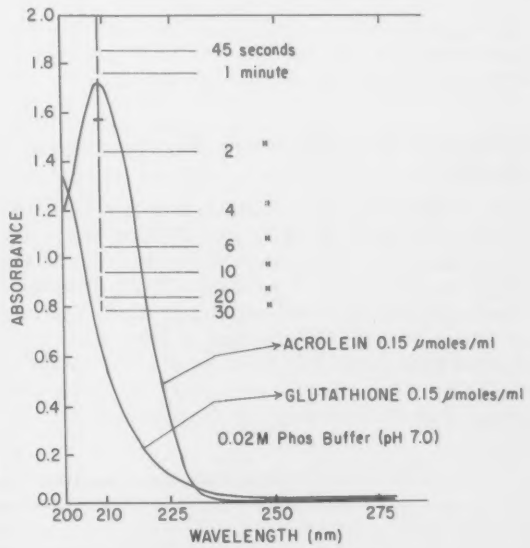


FIGURE 3.—Decrease of acrolein absorption at 209 nm following interaction with equimolar amounts of glutathione.

TABLE 2.—Decrease of acrolein's absorbance by sulfhydryl compounds*

	Time period (secs) in which 50% decrease of the 209-nm absorption peak occurred		
	pH 7	pH 7.4	pH 8.5
Glutathione	111	58	17
Acetylcysteine	345	165	57
Cysteine	74	30	4-5

*Reactions carried out at room temperature (23°C-25°C). Concentration of reactants, 0.15 μmol/ml 0.02 M phosphate buffer (Na_2HPO_4-KH_2PO_4).

TABLE 3.—Decrease of acrolein's absorbance by some amino acids*

	Decrease of 209 nm peak (%)	Time incubated at room temperature (mins)
Glycine	10	40
Arginine	8	45
Serine	2	45
Methionine	0	15

*Concentration of acrolein and amino acids, 0.15 μmol/ml 0.02 M phosphate buffer, pH 7.4.

pH values. The fastest reaction observed was the interaction of acrolein with cysteine at pH 8.5. In interactions of acrolein with amino acids with other functional groups, the decrease in absorbance was very slow (table 3).

Iodometric titration of the sulfhydryl compounds (25) before and after interaction with acrolein further confirmed that the reaction took place at the sulfhydryl group site in these compounds.

Adduct Decomposition With Regeneration of Acrolein

It is assumed that conjugation of acrolein with glutathione takes place by the addition of the nucleophilic glutathione anion to the β carbon of the allylic double bond of acrolein (20,31). Reactions where glutathione is substituted for acetylcysteine or cysteine would be expected to take place by a similar mechanism. The products of these reactions would be 3-substituted propanals, a well-known group of acrolein-generating compounds (7,27).

Following procedures formerly used to collect acrolein during in vitro oxidation of spermine or CP (1,6), the above adducts, incubated at 37°C and 100°C, similarly generated this aldehyde. Air was used as the carrier gas to remove the regenerated acrolein into cold-water traps where it was collected by absorption. In table 4 are listed the amounts of acrolein collected from these incubations during two time periods at each temperature. In the experiments at 100°C all the acrolein that could be recovered (83% yield) was collected during the first 40 minutes. Reference solutions of acrolein produced 88% yield under similar experimental conditions. The 2-hour periods in the incubations at 37°C were arbitrarily chosen and the amounts of acrolein collected do not represent the maximal amounts that can be obtained at these temperatures.

3-Substituted propanals can be stabilized by their reduction to the corresponding alcohols. This procedure was employed for the identification of the aminoaldehydes of spermine, using sodium borohydride as the reducing agent (32). A similar method was applied to the acrolein-acetylcysteine and acrolein-cysteine adducts. The expected alcohols, if 3-substituted propanals had been formed, were MCA and 3-H Pr Cyst respectively, ie, the same compounds already obtained from the urine of rats given acrolein. This did occur, and both compounds were detected by paper chromatographic techniques and quantitated with the amino-acid analyzer. Preliminary measurements indicate that reduction of the acrolein-acetylcysteine adduct resulted in the formation of MCA in a 49.6% yield, and reduction of the acrolein-cysteine adduct re-

TABLE 4.—Acrolein generation from acrolein-sulfhydryl compound adducts*

	Temperature				
	37°C		100°C		
Adduct	Incubation period (hrs)	Acrolein collected† (μmols)	Incubation period (mins)	Acrolein collected† (μmols)	Maximal yields (%)
Glutathione-acrolein	1	0.55	10	7.50	
	2	1.20	40	12.30	82
Acetylcysteine-acrolein	1	0.13	10	5.65	
	2	0.44	40	12.40	83
Cysteine-acrolein	1	3.20	10	7.40	
	2	5.50	40	12.50	83
Acrolein (buffered reference solution)	1	7.10	10	11.50	
	2	9.80	40	13.20	88

*Adduct formation = 15 μmols of each compound in 100 ml of 0.02 M phosphate buffer (pH 7.4), incubated at room temperature (23°C-25°C) for 1-2 hrs.

†Carrier gas = air 130-140 ml/min. Acrolein collected in 150-ml cold-water traps.

sulted in formation of 3-H Pr Cyst in a 21.6% yield. If further confirmed, these results would indicate that the addition reaction to acrolein's double bond took place, but that at the same time another form of reversible reaction, judging from the 83% recovery of acrolein from the original adducts, most likely occurred. The glutathione acrolein adduct has not yet been similarly studied.

DISCUSSION

This paper on the identification and quantitation of MCA in the urine of rats given CP, related drugs, allyl alcohol, or acrolein, added to the basic work by Kaye's group (17-20) and Gray and Barnsley (21), and to the physiopathologic studies on the hepatotoxicity of allyl alcohol (33-35), led, with a high degree of certainty, to the conclusion that acrolein is generated in vivo from all these compounds as it is in vitro. Acrolein, as already considered by others (20,21), appears to be the required intermediate for the above MCA formation.

Consequently, MCA presence in the urine of rats or man given CP indicates that the active metabolite aldophosphamide decomposes in vivo with the generation of acrolein, as it does in vitro (3-5,27). Thus, the levels of this MCA will be directly proportional to the fraction of aldophosphamide that escaped inactivation to carboxyphosphamide, as well as to the amount of phosphorodiamidic acid mustard formed. Structurally, this latter active metabolite can be considered as the residual part of aldophosphamide once acrolein is eliminated. It follows that the MCA quantitation indirectly measures "active CP" (fig 4). This is an important consideration since methods most commonly used to follow CP activation in vivo and attempts to assess potential pharmacologic modifications of this activation have relied heavily on the nonspecific alkylation of 4-(*p*-nitrobenzyl)pyridine (36), with alkylation values usually expressed in relation to a standard, such as *nor*-nitrogen mustard (37).

There is an awareness among investigators that the diverse alkylating metabolites derived from CP may possess different molecular extinction coefficients, that the alkylation values obtained are the sum of the individual ones, and more important, that alkylating activity does not necessarily indicate antitumor or cell growth inhibitory activities. For example, although carboxyphosphamide, in itself, does not possess significant antitumor activities and apparently is nontoxic in vivo, nevertheless, it is as good an alkylating agent as aldophosphamide (37). This latter property makes it difficult to estimate the aldophosphamide to carboxyphosphamide ratio in any given alkylation measurements in blood samples or other organic fluids. This has been a serious limitation to many studies attempting to induce or inhibit CP activation. For example, it is known that the total concentration of alkylating activity in the blood (C) multiplied by time (T) remained relatively constant for a given CP dosage. In one clinical study where a deliberate attempt was made to potentiate CP activity in one patient by concurrent administration of an aldehyde oxidase inhibitor, the C × T product obtained was not higher than that of other patients receiving the same doses of CP (38). This observation, unfortunately, appears to have discouraged further clinical studies along this line of research. The above results were to be expected since C includes the alkylating activity of aldophosphamide, carboxyphosphamide, and others. Consequently, even if the drug combination succeeded in increasing the aldophosphamide to carboxyphosphamide ratio, the alkylation measurements alone could not have detected it. MCA quantitation in this patient's urine, on the other hand, might have detected this change as suggested by the experimental data presented in this paper, a possibility which,

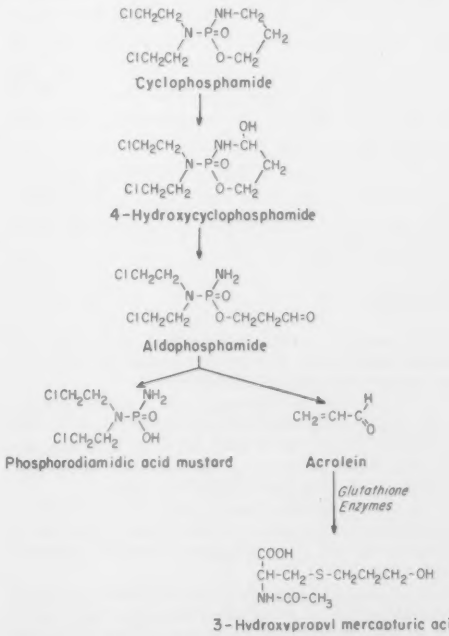


FIGURE 4.—Schematic representation of the main active metabolic pathway of CP with one of its end products, acrolein, as the MCA precursor.

together with that of the presence of MCA precursors in the blood, will be subsequently investigated.

Speculations on the mechanism of action of the acrolein formed in vivo or administered suggest that compounds possessing accessible sulfhydryl groups might be acrolein's main target. The reaction rate of acrolein with these compounds, as observed in vitro, is far above that with compounds possessing amino or hydroxyl groups. Barring unexpected enzymatic catalyses, it appears that this modality of action might also take place in vivo. The reaction of acrolein with available sulfhydryl groups of enzymes has been described (39, 40), as well as the decrease of acrolein cytotoxicity in vitro by glutathione (41). It is most likely that glutathione in the cells is the main (although not necessarily the most significant) target of acrolein, judging from (*a*) the resulting MCA yields, (*b*) the glutathione ubiquity and concentration in diverse tissues (42,43), (*c*) the existence of glutathione alkene-transferase (31) which might catalyze in vivo this already fast interaction, and (*d*) the decrease in glutathione levels in the liver and kidney of experimental animals given acrolein as observed by us in preliminary experiments.

Considering the large variations of MCA excretion in the few patients studied so far, possible correlations that might be responsible for these variations will be searched for as soon as a significantly large number of patients receiving CP are studied. Since the patients reported here received multiple-drug therapy, it is conceivable that the highest levels measured might be the result of a non-planned stimulation of CP activation. Because of the small number of observations it is not possible at this moment to advance an explanation for the MCA values found.

REFERENCES

1. Alarcon RA, and Meienhofer J. Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide. Nature [New Biol] 233:250-252, 1971.
2. Colvin M, Padgett CA, and Fenselau C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
3. van der Steen J, Timmer EC, Westra JG, et al. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
4. Connors TA, Cox PJ, Farmer PB, et al. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
5. Voelcker G, Draeger U, Peter G, et al. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
6. Alarcon RA. IV. Evidence for the formation of the cytotoxic aldehyde acrolein from enzymatically oxidized spermine or spermidine. Arch Biochem Biophys 137:365-372, 1970.
7. Li PK. Studies on the mode of action of bovine plasma spermine oxidase. PhD thesis, Northwestern University, Evanston, Ill, 1971.
8. Kimes BW, and Morris DR. Preparation and stability of oxidized polyamines. Biochim Biophys Acta 228:223-234, 1971.
9. Brock N, and Hohorst H-J. Metabolism of cyclophosphamide. Cancer 20:900-904, 1967.
10. Norpoth K, Golovinsky E, and Rauen HM. Untersuchungen an hypothetischen Metaboliten des 2-[Bis(2-chlorethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-oxids (Cyclophosphamid). Hoppe Seylers Z Physiol Chem 351:377-383, 1970.
11. Friedman OM, Boger E, Grubliauskas V, et al. Synthesis of *N*-phosphorylated derivatives of nitrogen mustards with latent cytotoxicity. J Med Chem 6:50-58, 1963.
12. Maddock CL, Handler AH, Friedman OM, et al. Primary evaluation of alkylating agent cyclohexylamine salt of *N,N*-bis(2-chlorethyl)phosphorodiamidic acid (NSC-69945, OMF-59) in experimental antitumor assay systems. Cancer Chemother Rep 50:629-639, 1966.
13. Nathanson L, Hall TC, Rutenberg A, et al. Clinical toxicologic study of cyclohexylamine salt *N,N*-bis(2-chloroethyl)-phosphorodiamidic acid (NSC-69945; OMF-59). Cancer Chemother Rep 51:35-39, 1967.
14. Hill DL, Laster WR, and Struck RF. Enzymatic metabolism of cyclophosphamide and nicotine and production of a toxic cyclophosphamide metabolite. Cancer Res 32:658-665, 1972.
15. Struck RF. Isolation and identification of a stabilized derivative of aldophosphamide, a major metabolite of cyclophosphamide. Cancer Res 34:2933-2935, 1974.
16. Fenselau C, Kan MN, Billets S, et al. Identification of phosphorodiamidic acid mustard as a human metabolite of cyclophosphamide. Cancer Res 35:1453-1457, 1975.
17. Clapp JJ, Kaye CM, and Young L. Observations on the metabolism of allyl compounds in the rat. Biochem J 114:6P-7P, 1969.
18. Kaye CM, and Young L. Mercapturic acid formation from allyl compounds in the rat. Biochem J 119:53P, 1970.
19. ——. The synthesis of mercapturic acids from allyl compounds in the rat. Biochem J 127:87P, 1972.
20. Kaye CM. Biosynthesis of mercapturic acids from allyl alcohol, allyl esters and acrolein. Biochem J 134:1093-1101, 1973.
21. Gray JM, and Barnsley EA. The metabolism of crotyl phosphate, crotyl alcohol and crotonaldehyde. Xenobiotica 1:55-67, 1971.
22. Kaye CM, and Young L. Acrolein as a possible metabolite of cyclophosphamide in man. Biochem Soc Trans 2:308-310, 1974.
23. Barnsley EA. The formation of 2-hydroxypropylmercapturic acid from 1-halogenopropanes in the rat. Biochem J 100:362-372, 1966.
24. Kaye CM, Clapp JJ, and Young L. The metabolic formation of mercapturic acids from allyl halides. Xenobiotica 2:129-139, 1972.
25. Woodward GE, and Fry EG. The determination of blood glutathione. J Biol Chem 97:465-482, 1932.
26. Alarcon RA. Fluorometric determination of acrolein and related compounds with *m*-aminophenol. Anal Chem 40:1704-1708, 1968.
27. Alarcon RA, Meienhofer J, and Atherton E. Isophosphamide as a new acrolein-producing antineoplastic isomer of cyclophosphamide. Cancer Res 32:2519-2523, 1972.
28. Toennies G, and Kolb JJ. Techniques and reagents for paper chromatography. Anal Chem 23:823-826, 1951.
29. Benson JV, and Patterson JA. Accelerated chromatographic analysis of amino acids commonly found in physiological fluids on a spherical resin of specific design. Anal Biochem 13:265-280, 1965.
30. Boyland E, and Chasseaud LF. The role of glutathione and glutathione *S*-transferases in mercapturic acid biosynthesis. Adv Enzymol 32:173-219, 1969.

31. ——. Enzyme-catalyzed conjugations of glutathione with unsaturated compounds. Biochem J 104:95-102, 1967.
32. Tabor CW, Tabor H, and Bachrach U. Identification of the aminoaldehydes produced by the oxidation of spermine and spermidine with purified plasma amine oxidase. J Biol Chem 239:2194-2203, 1964.
33. Rees WD, and Tarlow MJ. The hepatotoxic action of allyl formate. Biochem J 104:757-761, 1967.
34. Reid WD. Mechanisms of allyl alcohol-induced hepatic necrosis. Experientia 28:1058-1061, 1972.
35. Serrafini-Cessi F. Conversions of allyl alcohol into acrolein by rat liver. Biochem J 128:1103-1107, 1972.
36. Friedman OM, and Boger E. Colorimetric estimation of nitrogen mustards in aqueous media. Anal Chem 33:906-910, 1961.
37. Sladek NE. Bioassay and relative cytotoxic potency of cyclophosphamide metabolites generated in vitro and in vivo. Cancer Res 33:1150-1158, 1973.
38. Bagley CM, Bostick FW, and DeVita VT, Jr. Clinical pharmacology of cyclophosphamide. Cancer Res 33:226-233, 1973.
39. Munsch N, Recondo AM, and Frayssinet C. In vitro binding of ^{3}H-acrolein to regenerating rat liver DNA polymerase. Experientia 30:1234-1236, 1974.
40. Rando RR. Allyl alcohol-induced irreversible inhibition of yeast alcohol dehydrogenase. Biochem Pharmacol 23:2328-2331, 1974.
41. Alarcon RA. Comparison of the effects of oxidized spermine and acrolein on mammalian cells. Fed Proc 25:733, 1966.
42. Knox WE. Glutathione. *In* The Enzymes, 2nd ed (Boyer PD, Lardy H, and Myrbäck K, eds). New York, Academic Press, Inc, 1960, vol 2, pp 253-294.
43. Flohé L, Benöhr HCH, Sies H, et al, eds. Glutathione. New York, Academic Press, Inc, 1974.

Cyclophosphamide (NSC-26271)-Related Phosphoramide Mustards—Recent Advances and Historical Perspective[1,2]

Orrie M. Friedman,[3,*] Isidore Wodinsky,[4] and Arthur Myles[3]

SUMMARY

The antitumor activity of three of the most active phosphoramide mustards, NSC-69947, NSC-72505, and NSC-72510, was compared with that of phosphoramide mustard (NSC-69945), the apparent active metabolite of cyclophosphamide (CP), against the L1210, P388, B16, and Lewis lung tumor systems. This comparison did not reveal any significant differences in the patterns of inhibitory activity predictive of significant advantages in the clinic of any of these compounds over CP or NSC-69945. Attempts to prepare aldophosphamide, the key intermediate in CP metabolism, by oxidation of hydroxyphosphamide under the Sarett reaction conditions lead primarily to 4-ketocyclophosphamide. Under milder conditions the product isolated appears to be the elusive aldophosphamide on the basis of positive alkylating and aldehyde tests, R_f and infrared data, and the formation and characterization of a semicarbazone. The possibility that this product is an equilibrium mixture with the tautomeric 4-hydroxycyclophosphamide has not been as yet definitely ruled out. Sarett oxidation of homohydroxyphosphamide straightforwardly gives the stable analog, homoaldophosphamide. Biologic testing of this putative aldophosphamide in direct comparison with homoaldophosphamide, CP, and NSC-69945 reveals that aldophosphamide is a potent antitumor agent indistinguishable in activity from CP and NSC-69945, whereas homoaldophosphamide is inactive. These results provide confirmatory evidence for the postulated role of aldophosphamide as an intermediate in CP metabolism and suggest, furthermore, that aldophosphamide itself is not active in vivo, requiring transformation to NSC-69945 via β-elimination of acrolein to exert its antitumor effects. An historical account of the development of the phosphoramide mustard field is also given.

[Cancer Treat Rep 60:337-346, 1976]

This would seem an opportune time to review developments in the phosphoramide mustard field, particularly the current status of cyclophosphamide (CP) metabolism, because substantial progress is now being made towards understanding the physiologic fate and mechanism of action of this extremely useful drug. Hopefully, exploration and exploitation of the new insights that may be gained will lead to the development of a new generation of CP-related antineoplastic agents with superior chemotherapeutic potentialities.

PHOSPHORAMIDE MUSTARDS

Our interest in the phosphoramide mustards, of which CP is an important representative, began in 1948 when a group associated with Dr. Arnold Seligman set about to fashion rational cancer chemotherapy based on the use of latent cytotoxic agents (1). Our interests centered on the potentialities of the phosphoramide mustards with the hope that we could devise derivatives that would be activated in tumor cells by the enzyme, phosphamidase, described by Gomori (2) as being abundant in

[1]Supported by contracts N01-CM-60506 and N01-CM-53770 from the Division of Cancer Treatment, National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Collaborative Research, Inc, Waltham, Mass.

[4]Arthur D. Little, Inc, Cambridge, Mass.

*Reprint requests to: Dr. Orrie M. Friedman, Collaborative Research, Inc, 1365 Main St, Waltham, Mass 02154.

certain human neoplasms. Over a period of time, we synthesized a considerable number of new phosphoramide mustards (3-9) of four general structural types—(A) diamidic esters, (B) triamides, (C) esteramidic acids, and (D) diamidic acids (*see* fig 1)—as enzyme-activatable biologic carriers of nitrogen mustards that might have selective toxicity for tumors.

Attempts were initially made to study these compounds enzymatically but these efforts were abandoned because of the technical difficulties involved. The compounds were eventually tested for antitumor activity, first at the Sidney Farber Cancer Center (formerly the Children's Cancer Research Foundation), Boston, Mass (10), and later at Arthur D. Little, Inc, Cambridge, Mass, under the NCI's testing program. The results of this latter testing, reported in detail elsewhere (9), indicated generally that the tri-substituted derivatives in categories A and B tended to be low in both cytotoxicity and antitumor activity, whereas the disubstituted monoanionic derivatives in categories C and D were more cytotoxic and showed a much greater incidence of high levels of antitumor activity.

Biologic Testing

The testing data on four of the most active compounds in the series, the diamidic acid NSC-69945 (phosphoramide mustard), the aziridinyl triamide NSC-69947, the *t*-butyl amidic acid NSC-72505, and the aziridinyl amidic acid NSC-72510, compared to that of CP are summarized in table 1. In addition, a direct comparison was made of the activity of

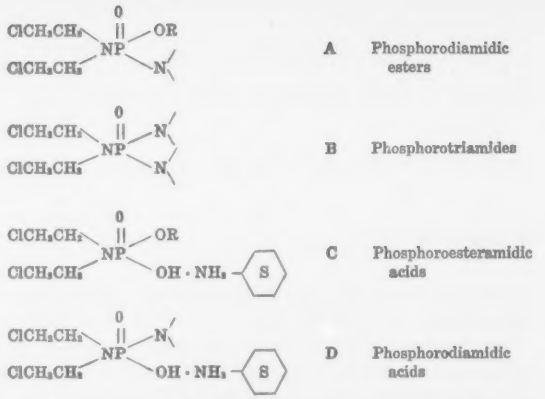


FIGURE 1.—The 4 general structural types of phosphoramide mustards.

NSC-69945, NSC-72505, and NSC-72510 with that of CP in a wide spectrum of tumors. The results (table 2) showed that NSC-69945 was active in 25 of 25 tumor systems tested, NSC-72505 in 34 of 34, and NSC-72510 in 33 of 34; CP was active in 30 of 35. It is interesting to note that NSC-69945 now appears to be the therapeutically effective metabolite of CP. Since NSC-69945 is one of the original phosphoramide mustards (4), this would seem a classic case of the synthesis of the active species preceding the conception of the drug, ie, an active metabolite in search of a latent precursor.

NSC-69945 is currently being clinically tested at the Sidney Farber Cancer Center, Boston, Mass. The other three active phosphoramide mustards (in

TABLE 1.—Comparison of biologic testing data of the 4 most active phosphoramide mustards with those of CP

NSC No.	X	Y	Cytotoxicity, KB cells (ED50, μg/ml)	Therapeutic response: Dunning ascites leukemia*	Therapeutic response: Dunning ascites leukemia/CP resistant†	Therapeutic response: L1210 leukemia†
72505	–OH·CHA	$-OC(CH_3)_3$	1.25	16	4	4
69945	–OH·CHA	$-NH_2$	3.3	64	2	4
72510	–OH·CHA		0.12	125	4	4
69947	$-NH_2$		0.2	16	2	4
CP	$-NHCH_2CH_2CH_2O-$		> 1000	10	—	2

*Curative range factor = highest dose yielding 67% or more "cures" at 30 days / lowest dose yielding 67% or more "cures" at 30 days

†Therapeutic range factor = highest dose producing 25% increase in survival time / lowest dose producing 25% increase in survival time

TABLE 2.—Comparison of the activity of CP and 3 CP analogs in a spectrum of tumors

Tumor and tumor-resistant strains	% T/C for—			
		CP analogs		
	CP	69945	72505	72510
L1210	169	185	156	175
L1210/MTX	159	—	183	193
L1210/38280	183	185	160	150
L1210/63878	177	227	—	—
P388	>237	172	>300	>300
P388/38280	174	226	127	129
P388/53306	127	—	135	156
P388/57155	>300	>266	>275	231
P388/VCR	157	246	142	200
P1534/CDBA	171	154	126	150
P1534J	108	227	>300	>300
$P1534J_{(s)}^{(n)}$	127	157	140	196
AK_4	>257	161	144	116
Mecca	200	158	125	127
Gardner	>206	>226	125	151
P329	113	153	146	137
Lymphoma 4	122	—	165	162
P815	152	164	210	221
P815/VBL	193	>300	188	225
P815/53306	162	>300	161	138
P815/MGGH	139	156	156	194
C1498	162	—	155	128
Lymphoma 2	225	>300	>300	216
RCS	157	—	226	156
L5178Y	131	185	142	139
$L5178Y_F$	—	—	196	125
P1081	121	—	>215	>243
P288	251	150	157	184
P288/MTX	135	—	155	175
Dunning Schmidt	>300	>300	>300	>300
Dunning ADL	>300	>300	>300	>300
Dunning/CP	110	131	150	165
Dunning/29189	>300	—	>300	>300
P1798*	28	27	38	37
P1798/CR*	40	—	7	27
P1798/FHCR*	40	—	—	—
No. of active tumor systems/ No. of tumor systems tested	30/35	25/25	34/34	33/34

*T/C for tumor inhibition.

a sense, analogs of NSC-69945) are possible candidates for preclinical toxicology and pharmacology studies. These three compounds (NSC-69947, NSC-72505, and NSC-72510) were recently resynthesized for direct comparison with NSC-69945 and CP in a variety of tumor systems to determine which, if any, should go into clinical study.

The test data on the four experimental tumor systems studied to date, L1210 leukemia, P388 leukemia, B16 melanoma, and Lewis lung carcinoma, are summarized in table 3. These results generally confirm the high levels of antitumor activity that these compounds previously had shown in the Dunning ascites, Dunning ascites/CP resistant, and L1210 leukemia tumor systems (9) (table 1). The aziridinyl acid NSC-72510 appeared to be the most active of the five compounds tested followed by NSC-69945 and CP which showed very similar levels of activity. The *t*-butyl acid NSC-72505 was somewhat less active than the preceding compounds and the aziridinylamide NSC-69947 was the least active of all. The differences in many cases, however, are small and there is some question as to whether all of these differences are significant.

In individual tumor systems, NSC-72510 showed the highest activity of all of the compounds tested

TABLE 3.—Comparative antitumor activity of selected phosphoramide mustards against experimental tumors in mice

NSC No.	% ILS* for animals treated with single ip doses on— Day 1 only	Days 1 and 9	Days 1, 5, and 9	Days 1-9
		L1210 leukemia		
69947	41	64	105 (2/6)	147 (2/6)
72505	24	36†	18	70 (1/6)
72510	162 (4/6)	133 (2/6)	147 (2/6)	154 (4/6)
69945	165 (5/6)	46	106 (1/6)	87 (1/6)
CP	108 (3/6)	144 (1/6)	104 (1/6)	129 (1/6)
		P388 leukemia		
69947	68	141† (1/6)	126†	124 (1/6)
72505	156‡ (2/6)	161	156 (3/6)	156 (3/6)
72510	162 (3/6)	171 (6/6)	158 (5/6)	156† (3/6)
69945	164 (4/6)	123 (1/6)	158 (5/6)	152 (2/6)
CP	178 (3/6)	183 (5/6)	170 (6/6)	170 (4/6)
		B16 melanoma		
69947	24	34	37	17
72505	47	109	124	95
72510	58	94	86	80
69945	45	131	84	82
CP	54	60	24	38

Lewis lung carcinoma

	Day 8 only	Days 8 and 16	Days 8, 12, and 16	Days 8-16
69947	14	20	13	40
72505	1	13	19	18
72510	8	41	30	25
69945	39	30	50	55
CP	60	31	51	51

*Nos. in parentheses = No. of 30-day survivors/No. of mice inoculated.
†Lowest dose tested.
‡Highest dose tested.

against L1210 leukemia; NSC-69945 and CP were identical in their effect and showed the highest activity against the P388 tumor system. NSC-72505 was the most effective analog against B16 melanoma and CP was the most effective against the Lewis lung tumor system. There were many 30-day survivors ("cures") in the drug-treated L1210 and P388 mice. However, no dramatic quantitative or qualitative differences between the four agents have emerged from the testing studies thus far. Superficially at least, these compounds would appear to have more or less the same biologic mode of action.

Discussion

Since NSC-69945 now almost certainly appears to be the therapeutically effective metabolite of CP (11,12), it is of interest to investigate analogous structures not only as potentially useful anticancer agents but also as highly potent cytotoxic agents for which new highly selective latent precursor derivatives might be devised. Three of the most active analogs of NSC-69945 that have evolved thus far from our studies on the phosphoramide mustards, the aziridinyl triamide NSC-69947, the t-butyl ester acid NSC-72505, and the

aziridinyl acid NSC-72510, were re-evaluated as candidates for clinical study in four experimental tumor systems: L1210, P388, B16, and Lewis lung. While these test systems have confirmed the high level of antitumor activity of these compounds, the results do not provide a basis for discrimination since the four compounds showed a similar pattern of antitumor activity. What is needed is more precise information on the comparative effect of these compounds on macromolecular cellular metabolism as well as information on their molecular mechanism of action to provide a basis for selection for clinical study and possibly further use in drug combinations. In the meantime, work is in progress on the synthesis of a series of latent derivatives of these active phosphoramide mustards as analogs of aldophosphamide capable of undergoing β-elimination, with potentially superior chemotherapeutic potentialities.

CP METABOLITES

CP synthesized subsequently (13) on the basis of our phosphamidase rationale was conceived of as a "transport form" of *nor*-nitrogen mustard and has proven to be an extremely useful chemotherapeutic agent in the clinic. However, while CP is inactive per se and requires metabolism in vivo in order to become cytoactive, there is no evidence that such activation mediated by phosphamidase occurs in tumors. In fact Foley et al (14), studying CP metabolism in rats, discovered that liver is the principal site of CP activation. This observation was confirmed by Brock and Hohorst (15) who found in addition that the microsomal fraction is responsible and, moreover, that the process is oxidative rather than hydrolytic, requiring the presence of NADPH and oxygen.

Although the principal metabolic pathways for CP, including its activation in the liver (14), have now been well documented, the exact nature of the metabolite(s) responsible for its chemotherapeutic activity has not been fully clarified. It has been established that CP undergoes oxidative metabolism as outlined in figure 2 (11,15-21). Oxidation of CP (fig 2, I), α to the ring nitrogen, gives 4-hydroxycyclophosphamide (4-hydroxy-CP) (fig 2, II) or its tautomer aldophosphamide (fig 2, III). Further oxidation of this aldehyde III would give carboxyphosphamide (fig 2, V), whereas β-elimination of acrolein (fig 2, VIII) or hydrolytic cleavage of 3-hydroxypropionaldehyde (fig 2, VII) would lead to NSC-69945 (fig 2, VI). Further oxidation of 4-hydroxy-CP would give 4-ketocyclophosphamide (4-keto-CP) (fig 2, IV) or its hydrolysis product carboxyphosphamide.

The metabolic products referred to above, except for 3-hydroxypropionaldehyde (VII) and aldophosphamide or its hemiaminal tautomer 4-hydroxy-CP, have been isolated in vivo and characterized with respect to their chemical and biologic activity (11,16,19,22).

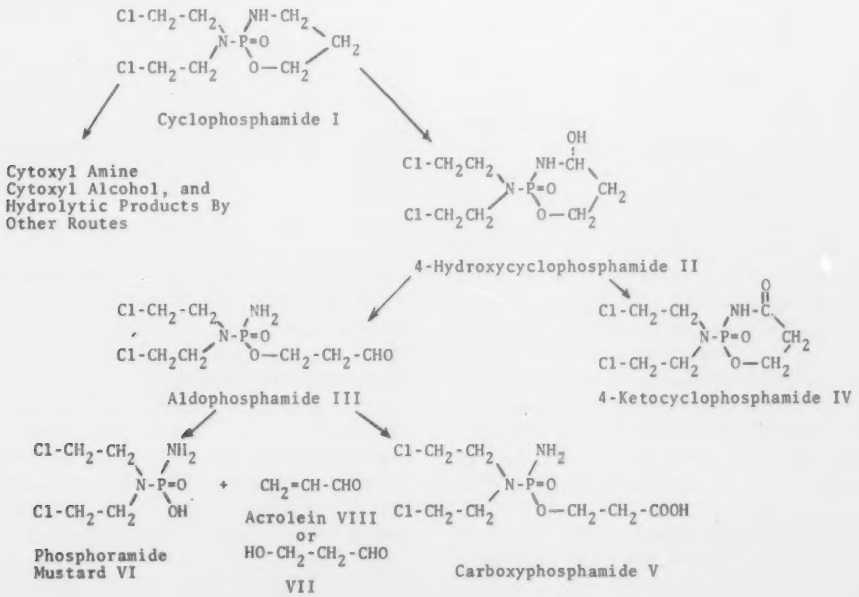


FIGURE 2.—Pathways of metabolism of CP.

Carboxyphosphamide and 4-keto-CP (the major metabolites of CP found in urine) have little or no cytotoxic or alkylating activity and are presumably detoxification products (23,24). In contrast, NSC-69945 is a potent alkylating agent with a high level of antitumor activity (9,10). It also shows a similarity to CP both in animal test systems and in preliminary clinical studies.[5] It has been isolated from liver microsomal oxidation of CP (12,19) and from patients treated with CP (11). NSC-69945 now appears to be the chemotherapeutically active metabolite of CP.

Verification of the presence of aldophosphamide or its tautomer 4-hydroxy-CP as a metabolite of CP in vivo has been the subject of considerable research. There is a great deal of evidence suggesting that aldophosphamide is a very labile compound which undergoes facile β-elimination to give NSC-69945 and acrolein (12,22,24-26). Most efforts to isolate and completely characterize this compound have been only partially successful although the crude unstable products isolated did appear to be cytotoxic (16,25-27) and in some cases also underwent a spontaneous decomposition to give acrolein and presumably NSC-69945 (26). Isolation of a stable semicarbazone derivative of aldophosphamide in vitro has recently been reported by Struck (28). It appears that this derivative is not as cytoactive as the crude aldophosphamide (24). There have been no reports on the isolation of the hydrolysis product of aldophosphamide, 3-hydroxypropionaldehyde (VII). This compound is known to polymerize or to dehydrate to give acrolein.

From what is now known of the metabolism of CP, it is clear that the production of NSC-69945 via aldophosphamide represents the principal therapeutically important metabolic pathway. A crucial question that still remains, however, is whether the intermediate aldophosphamide reaches tumor cells as such and is further metabolized intracellularly to a cytotoxic product(s), or whether it simply breaks down as it is formed in the liver and releases NSC-69945 into the circulation at that point. Our present efforts are directed at attempts to answer this question and to exploit information that may be gained about the mechanism of action of CP as a guide to the synthesis of new even more effective chemotherapeutic agents in this series.

Aldophosphamide is the last known or postulated principal metabolite of CP that remains to be synthesized. The availability of this elusive metabolite for biologic testing would be extremely useful in helping to clarify the role of this key postulated intermediate in the metabolism and antitumor activity of CP.

The preparation of aldophosphamide as a discrete isolable product, however, is expected to be difficult because of its anticipated chemical lability. It may rapidly equilibrate with 4-hydroxy-CP or readily decompose to NSC-69945 by β-elimination of acrolein. We report here our efforts to prepare aldophosphamide and certain stable analogs by an unambiguous practical route. As a model we used the preparation of the homologous homoaldophosphamide (fig 3 [XII], $n = 2$) which is stable because it is incapable of undergoing β-elimination.

The basic synthetic approach that we have pursued in the aldophosphamide series is the chemical oxidation of the corresponding alcohols XI to the respective aldehydes XII (fig 3). The phosphamide alcohols XI were readily prepared in quantity by standard procedures outlined in figure 3. A number of published oxidation procedures (26,29-33) were investigated using in some cases the conversion of a series of primary alcohols IX to the corresponding aldehydes X as a simple model system. In the two cases that have been most extensively investigated thus far (IX, $n = 1$ and 2), the Sarett procedure (33) produced a single major product which was both ultraviolet absorbing and gave a positive aldehyde test. Both products showed a carbonyl band of moderate intensity at 1725 cm^{-1} in the infrared (IR) characteristic of aliphatic aldehydes and both gave 2,4-dinitrophenylhydrazones with good analytic data. The other oxidation procedures tended to give small yields of the corresponding aldehyde, generally containing small amounts of several other ultraviolet and non-ultraviolet absorbing aldehydes as contaminants. From these results, the Sarett procedure appeared clearly the method of choice in attempts to synthesize more complex aldehydes.

Oxidation of homohydroxyphosphamide (fig 3 [XI], $n = 2$) resulted in the formation of two new products; of which the major product was an alkylating aldehyde as expected for the desired homoaldophosphamide (fig 3 [XII], $n = 2$). The IR spectrum of this product was similar to that of the starting homohydroxyphosphamide except for an additional peak in the carbonyl region at 1725 cm^{-1}. The product also gave a mass spectrum[6] entirely consistent with the homoaldophosphamide assignment of structure. Several hundred milligrams of this product have been prepared for biologic evaluation.

[5]Frei E, III. Personal communication.

[6]Colvin M, and Fenselau C. Unpublished data. Departments of Pharmacology and Medicine, Johns Hopkins University School of Medicine, Baltimore, Md.

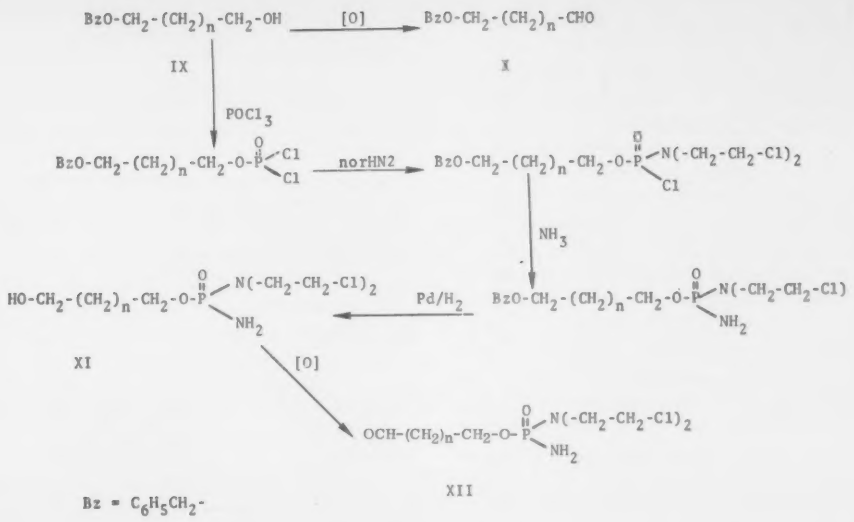


FIGURE 3.—Synthetic approach to the preparation of aldophosphamide and homoaldophosphamide.

Oxidation of hydroxyphosphamide (fig 3 [XI], n = 1) under Sarett oxidation conditions (33) gave a crystalline product which on the basis of melting point and analytic, IR, and mass spectral data was identical with 4-keto-CP. The desired aldehyde III apparently had formed initially but under the conditions of the reaction had equilibrated with the carbinolamine II which in turn was oxidized to the amide IV.

The reason that the corresponding keto compound is not formed in the case of the homologous aldehyde (fig 3 [XII], n = 2) is that it probably does not equilibrate to the corresponding cyclic carbinolamine because this involves cyclization to a sterically unfavored seven-membered ring structure.

Oxidation of hydroxyphosphamide (fig 3 [XI], n = 1) under milder conditions gave only traces of 4-keto-CP and led to the production of a mixture of products, the major constituent of which was a labile alkylating aldehyde with an R_f value essentially identical to that of the alkylating aldehyde derived from homohydroxyphosphamide. The corresponding phosphamide alcohols (fig 3 [XI], n = 1 and 2) have essentially identical but lower R_f values. This product also formed a semicarbazone in good yield with the correct elemental analysis for aldophosphamide semicarbazone, and the IR spectrum of this derivative was virtually superimposable on that of the semicarbazone of the corresponding homoaldophosphamide. These results are consistent with the assignment of the aldophosphamide structure to this product.

Attempts at mass spectral analysis were entirely negative, most likely because of the known instability of this compound under conditions of the determination. The sample probably liberates acrolein instantaneously leaving a residue of NSC-69945 which is not volatile without prior diazomethane treatment.

Also, IR analysis is not entirely consistent with the above assignment. While the IR spectrum resembles that of homoaldophosphamide over most of the range, it varied from sample to sample showing weaker absorption than expected in the carbonyl range at 1725 cm^{-1}, present in some cases only as a shoulder. The reason for this inconsistency is not entirely clear and may simply reflect the neighboring group effect of the amide in this particular structure. However, one cannot rule out the possibility that the product is an equilibrium mixture of the open-chain aldehyde and the cyclic 4-hydroxy-CP, in which the latter may predominate. From the point of view of biologic activity, this distinction between the two tautomeric forms may be academic if interconversion occurs readily under physiologic conditions giving the same equilibrium mixture regardless of the initial form.

Biologic Testing

Homoaldophosphamide and the putative aldophosphamide were tested in direct comparison with CP and NSC-69945 in vitro against the KB and L1210 cell lines in culture and in vivo against the L1210 tumor system in mice. The corresponding

alcohols (fig 3 [XI], $n = 1$ and 2) as well as 4-keto-CP were included in the study.

The results of the in vitro testing (summarized in table 4) indicate that the oxidation product of hydroxyphosphamide, "aldophosphamide" (fig 3 [XII], $n = 1$), is moderately cytotoxic in both cell systems at a level comparable with that of NSC-69945, whereas the homologous aldehyde, homoaldophosphamide (fig 3 [XII], $n = 2$), is somewhat less cytotoxic. The two alcohols (fig 3 [XI], $n = 1$ and 2) showed low levels of cytotoxicity and the activity of 4-keto-CP was comparable to that of NSC-69945. CP is, of course, not cytotoxic in vitro. While the pattern of results was parallel in both the KB and L1210 cell systems, the compounds were consistently more cytotoxic in the L1210 cell system. The 50% inhibitory dose (ID50) values, however, were generally higher and the differences between individual compounds smaller than might have been anticipated on the basis of earlier results with analogous compounds (9) for reasons that are not entirely clear.

The preliminary results of the in vivo testing against L1210 in mice, summarized in table 5, indicate that the putative aldophosphamide is a potent antitumor agent indistinguishable in activity from CP and NSC-69945, whereas homoaldophosphamide is essentially inactive. The two alcohols and 4-keto-CP, as anticipated, were devoid of antitumor activity.

Table 4.—In vitro cytotoxicity of aldophosphamide-related phosphoramide mustards

Compounds	ID50 (μg/ml) in— KB tumor cells	L1210 tumor cells
Homoaldophosphamide	12.5	5.6
Homohydroxyphosphamide	20.0	14.0
Hydroxyphosphamide	36.0	15.0
4-Keto-CP	11.0	4.0
Crude "aldophosphamide"	9.0	3.5
"Aldophosphamide"	9.0	4.0
NSC-69945	10.0	3.7

Discussion

The biologic testing of aldophosphamide in direct comparison with homoaldophosphamide, CP, and NSC-69945 was designed to help clarify the role of this elusive but important intermediate in the metabolism of CP.

Aldophosphamide and homoaldophosphamide are structurally phosphorodiamidic monoesters (fig 1, A) and by analogy to these compounds would not be expected to be either particularly cytotoxic or active as antitumor agents (9). However, the two homologs (fig 3 [XII], $n = 1$ and 2) contain an aldehyde group absent in all the other analogs in this series that might confer biologic activity. The lack of biologic activity of the stable homoaldophosphamide is consistent with the above structural expectation and indicates that the presence of the aldehyde function per se is unlikely to account for the biologic activity of aldophosphamide. One can reasonably infer from this that the unstable aldophosphamide is inactive in its native state, contrary to some earlier speculations (16,20,26,34), and that its high level of biologic activity is presumably related to its ability to degrade spontaneously to NSC-69945 and acrolein by β-elimination. This interpretation is not inconsistent with the possibility that the putative aldophosphamide that was studied is an equilibrium mixture with the tautomeric 4-hydroxy-CP. There is no theoretical reason

Table 5.—In vivo activity of aldophosphamide-related phosphoramide mustards on mice bearing L1210 leukemia*

Compounds†	Median survival time (days)	% ILS	No. of survivors on Day 20/ total No. of mice treated
Homoaldophosphamide	10.0	11	0/5
Homohydroxyphosphamide	9.0	0	0/5
Hydroxyphosphamide	9.0	0	0/5
4-Keto-CP	9.0	0	0/5
Crude "aldophosphamide"	> 27	> 200	5/5
"Aldophosphamide"	> 27	> 200	5/5
CP	> 27	> 200	4/5
NSC-69945	17.0	89	2/5
Control	9.0	0	0/40

*10^5 cells in 0.1 ml, ip.
†Administered as a single ip dose on Day 1 only.

to expect that the latter would be cytotoxic per se nor is there any evidence to support the possibility that it is.

The test results indicating that aldophosphamide, CP, and NSC-69945 are biologically indistinguishable are entirely consistent with the postulated role of aldophosphamide as a key intermediate in CP metabolism. Moreover, the data suggest that β-elimination takes place so rapidly that it effectively renders aldophosphamide and CP chemically equivalent to NSC-69945. This would imply (*a*) that the breakdown occurs in the liver, releasing NSC-69945 into the circulation at that point, and (*b*) that this hepatic transformation is essentially quantitative, which accounts for the dosage equivalence. This interpretation is inconsistent, however, with the several observations in animals and in man that the major detectable products of CP metabolism are carboxyphosphamide and 4-keto-CP, indicating, quantitatively at least, that the breakdown of aldophosphamide to NSC-69945 in vivo may be a minor metabolic pathway (35). Moreover, there is no information as yet on the blood levels and physiologic fate of NSC-69945 after ip injection of the drug. Thus, from the evidence available an assessment cannot be made of the precise role of aldophosphamide either as a metabolic intermediate to NSC-69945 in the liver or as a carrier to transport this active product to its site of action. Other studies designed to bear more specifically on these questions are now in progress.

REFERENCES

1. Seligman AM, Nachlas MM, Manheimer LH, et al. Development of new methods for the histochemical demonstration of hydrolytic intracellular enzymes in a program of cancer research. Ann Surg 130:333-341, 1949.
2. Gomori G. Histochemical demonstration of sites of phosphamidase activity. Proc Soc Exp Biol Med 69:407-409, 1948.
3. Friedman OM, and Seligman AM. Preparation of *N*-phosphorylated derivatives of bis β-chloroethylamine. J Am Chem Soc 76:655-661, 1954.
4. Friedman OM, Boger E, Grubliauskas V, et al. Synthesis of *N*-phosphorylated derivatives of nitrogen mustards with latent cytotoxicity. J Med Chem 6:50-58, 1963.
5. Friedman OM, Levi RS, Papanastassiou ZB, et al. Potential carcinolytic agents. I. Derivatives of *P,P*-bis(1-aziridinyl)-phosphinic amide. J Med Chem 6:449-450, 1963.
6. Friedman OM, Papanastassiou ZB, Levi RS, et al. Potential carcinolytic agents related to cyclophosphamide. J Med Chem 6:82-83, 1963.
7. Friedman OM, Grubliauskas V, and Wodinsky I. Comparative antitumor activities of series of new phosphoramide mustards. Proc Am Assoc Cancer Res 4:21, 1963.
8. Olsen G, and Friedman OM. Cyclohexylammonium hydrogen *N*-2-chloroethyl-*N*-5-chloro-(1-chloromethyl)pentyl phosphorodiamidate. J Med Chem 9:162, 1966.
9. Friedman OM. Studies on some newer phosphoramide mustards. Cancer Chemother Rep 51:347-357, 1967.
10. Maddock CL, Handler AH, Friedman OM, et al. Primary evaluation of alkylating agent cyclohexylamine salt of *N,N*-bis(2-chloroethyl) phosphorodiamidic acid (NSC-69945; OMF-59) in experimental antitumor assay systems. Cancer Chemother Rep 50:629-639, 1966.
11. Fenselau C, Kan M-NN, Billets S, et al. Identification of phosphorodiamidic acid mustard as a human metabolite of cyclophosphamide. Cancer Res 35:1453-1457, 1975.
12. Connors TA, Cox PJ, Farmer PB, et al. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
13. Arnold H, and Bourseaux F. Synthese und Abbau cytostatisch wirksamer cyclischer *N*-Phosphamidester des Bis-(β-chloräthyl)-amins. Angew Chem 70:539-544, 1958.
14. Foley GE, Friedman OM, and Drolet BP. Studies on the mechanism of action of Cytoxan. Evidence of activation in vivo and in vitro. Cancer Res 1:57-63, 1961.
15. Brock N, and Hohorst HJ. Uber die Aktivierung von Cyclophosphamid in vivo und in vitro. Arzneim Forsch 13:1021-1031, 1963.
16. Hill DL, Laster WR, Jr. and Struck RF. Enzymatic metabolism of cyclophosphamide and nicotine and production of a toxic cyclophosphamide metabolite. Cancer Res 32:658-665, 1972.
17. Connors TA, Foster AB, Gilsenan AM, et al. Observations on the metabolism of cyclophosphamide. Biochem Pharmacol 21:1373-1376, 1972.
18. Sladek NE. Evidence for an aldehyde possessing alkylating activity as the primary metabolite of cyclophosphamide. Cancer Res 33:651-658, 1973.
19. Colvin M, Padgett CA, and Fenselau C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
20. Hohorst HJ, Ziemann A, and Brock N. 4-Ketocyclophosphamide, a metabolite of cyclophosphamide. Formation, chemical and biological properties. Arzneim Forsch 21:1254-1257, 1971.
21. Bakke JE, Feil VJ, Fjelstul CE, et al. Metabolism of cyclophosphamide by sheep. J Agric Food Chem 20:384-388, 1972.
22. Alarcon RA, and Meienhofer J. Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide. Nature [New Biol] 233:250-252, 1971.
23. Struck RF, Kirk MC, Mellett LB, et al. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
24. Sladek NE. Bioassay and relative cytotoxic potency of cyclophosphamide metabolites generated in vitro and in vivo. Cancer Res 33:1150-1158, 1973.
25. Takamizawa A, Matsumoto S, Iwata T, et al. Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
26. van der Steen J, Timmer EC, Westra JG, et al. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
27. Struck RJ, Thorpe MC, Coburn WC, Jr. et al. Cyclophosphamide. Complete inhibition of murine leukemia L1210 in vivo by a Fenton oxidation product. J Am Chem Soc 96:313-315, 1974.
28. Struck RF. Isolation and identification of a stabilized derivative of aldophosphamide, a major metabolite of cyclophosphamide. Cancer Res 34:2933-2935, 1974.
29. Corey EJ, and Kim CU. Oxidation of primary and secondary alcohols to carbonyl compounds using dimethyl sulfoxide-chlorine complex as reagent. Tetrahedron Lett 12:919-922, 1973.
30. Corey EJ, and Fleet GWJ. Chromium trioxide-3,5-dimethylpyrazole complex as a reagent for oxidation of alcohols to

carbonyl compounds. Tetrahedron Lett 45:4499-4501, 1973.
31. Gritter RJ, and Wallace TJ. The manganese dioxide oxidation of allylic alcohols. J Org Chem 24:1051-1056, 1959.
32. Lalancette JM, Rollin G, and Dumas P. Metals intercalated in graphite. I. Reduction and oxidation. Can J Chem 50:3058-3062, 1972.
33. Ratcliffe R, and Rodehorst R. Improved procedure for oxidations with chromium trioxide-pyridine complex. J Org Chem 35:4000-4001, 1970.
34. Norpoth K, Golovinsky E, and Rauen HM. Untersuchungen an hypothetische Metaboliten des 2-[Bis(2-chloräthyl)-amino)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-oxids (Cyclophosphamid). Hoppe-Seylers Z Physiol Chem 351:377-383, 1970.
35. Torkelson AR, LaBudde JA, and Weikel J. The metabolic fate of cyclophosphamide. Drug Metab Rev 3:131-165, 1974.

Cyclic Phosphoramide Mustard (NSC-69945) Derivatives of Amino Acids and Peptides [1,2]

M. Szekerke[3,4,*]

SUMMARY

The possibilities offered by a combination of the "latency" and "carrier" principles were studied by joining the phosphoramide mustard group with amino acids. The structures of the resulting derivatives are reminiscent of cyclophosphamide, but they provide feasibility for stereoisomeric modifications and for incorporation into synthetic peptides. Model peptides with a different ring system containing cyclic diesters of the *N,N*-bis(2-chloroethyl)phosphoramidic acid were also constructed. The relative intensities of antitumor activity, toxicity, and antitumor spectrum can be influenced by these molecular manipulations, indicating that the pharmacokinetics, and probably even the enzymatic activation, are markedly dependent upon the overall physicochemical properties and stereochemistry of the molecule. Another approach has been to use larger molecular weight materials as carriers either of the phosphoramide mustard group or the cyclic amino acid derivatives. Both noncovalent and covalent linkages were tried; based on stability properties, conjugates must be regarded as more adequate potential candidates for lysosomotropism.

[Cancer Treat Rep 60:347-354, 1976]

The importance of cyclophosphamide (CP) (a representative of latent mustards) as an antitumor drug has suggested the search for CP analogs with the aim of widening the spectra of activity and diminishing the side effects. An important argument in favor of such studies is that quite often, information gained from the analysis of structure-activity relationships has helped explain the mechanism of action of the drug.

Our aim was to study the possibilities offered by a combination of the "latency" and "carrier" principles. The phosphoramide mustard group was regarded as a fixed biofunctional moiety considered essential for biologic activity. The six-membered ring system of CP was supposed to be a disposable biofunctional moiety which was temporarily a part of the drug molecule and was dismembered, at least partially, upon formation of the active compound. As a rule, in the disposable moiety there is a larger degree of freedom permissible with regard to molecular manipulations.

Amino Acid Carriers

A possible way to modulate the distribution of biologically active compounds is by using selective moieties, such as amino acids, sugars, etc, which are actively transported in the body by specific transport mechanisms. Ascites tumor cells, for instance, are reported to have a high capacity for the uptake of amino acids and sugars. Ring systems incorporating the phosphoramide mustard group were therefore constructed with the aid of

---

[1]Supported in part by the CHINOIN Pharmaceutical Works.

[2]Phosphoramide mustard: CAS reg. No. 1566-15-0; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-, compd. with cyclohexylamine.

[3]Institute of Organic Chemistry, Eötvös University, Budapest, Hungary.

[4]I wish to thank Prof. V. Bruckner for his continued interest in this work, Dr. J. Császár and M. T. Kajtár for their contributions to the synthetic work in the early stages of this research, and Mrs. G. Németh for her outstanding technical assistance. I am indebted to Dr. T. A. Connors, his co-workers, and many other staff members of the Chester Beatty Research Institute (London, England) for their invaluable help and support of this work for many years. Thanks are expressed for cooperation and for permission to quote biologic data from Profs. J. Baló and K. Lapis, and Dr. B. Szende, 1st Institute of Pathology, Semmelweis Medical University (Budapest), and Dr. L. Németh et al, Oncopathological Research Institute (Budapest).

*Reprint requests to: Dr. M. Szekerke, Institute of Organic Chemistry, Eötvös University, Budapest 1088, Hungary.

amino acids which could possibly function as selective conducting moieties.

The following hydroxyamino acids, or their esters, were used to achieve this end: DL-, L-, and D-serine (1,2), *threo*-DL-β-phenyl-serine, *erythro*-DL-β-phenyl-serine, *threo*-DL-β-hydroxy-glutamic acid (3), and *threo*-DL-β-(*p*-nitrophenyl)-serine (4) (table 1). The five-membered ring system is analogous with norendoxan (B 485) (5) rather than with CP, but there is a broader scope for the introduction of substituents and also for stereoisomeric modifications.

Somewhat different ring systems were obtained by the use of the following trifunctional amino acids: DL-lysine, L-cysteine (3), and L-tyrosine (4) (fig 1).

Screening data for the compounds depicted in table 1 are summarized in table 2 (6).[5] The DL-lysine and L-cysteine derivatives proved to be too toxic. Results of animal tests for the L-tyrosine ethyl ester derivative are also included in table 2.

Peptide Carriers

Encouraging previous results of melphalan and sarcolysin peptides with respect to reduced toxicity (7-9) prompted us to extend the work in this line and synthesize peptide derivatives of the cyclic phosphoramide mustards (table 1) obtained from hydroxyamino acids. One possibility inherent in this structure is to use the free carboxyl group of the original amino acid for coupling via a peptide bond with the amino group of a second amino acid not involved in the formation of a cyclic phosphoramide mustard (4). The synthetic compounds corresponding to this structure are shown in figure 2.

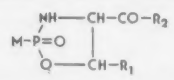
The second type of peptide model contained the fixed moiety incorporated into a different and larger ring. The two hydroxyl groups in a protected dipeptide composed of two hydroxyamino acids were coupled with *N,N*-bis(2-chloroethyl)phosphoramidic dichloride resulting in cyclic diesters of *N,N*-bis(2-chloroethyl)phosphoramidic acid (1,2,4). Compounds synthesized following this pattern are summarized in table 3. Screening data obtained for the peptide derivatives can be found in tables 4-6.[6]

DISCUSSION

Evaluation of the biologic tests is complicated by the fact that there are no data available for all compounds in at least one identical tumor system. Comparison of derivatives tested in the ADJ/PC6A plasma-cell tumor and in the Walker 256 carcinosarcoma can be based on the 50% lethal dose (LD50), 90% inhibitory dose (ID90), and therapeutic index (TI) (LD50/ID90) values. In the other systems only one or two dose levels were used, therefore these tests are more suitable as indications for the activity spectrum or toxicity differences. The methods by which these animal tests were carried out have been reported elsewhere (10-12). Different screeners used various methods to overcome the difficulties caused by the disadvantage that many of the derivatives are insoluble in water. Compounds were given ip in oil, ip in 10% ethanol-oil, and by solubilization with Tween 80.

The first objective of our study was to determine the effect of chirality on biologic activity. The L- and D-serine derivatives (table 2) showed almost identical inhibition of the Yoshida sc sarcoma at identical dose levels, but figures for toxicity and TI are not available.

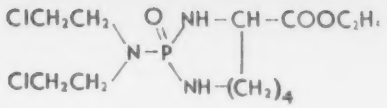
TABLE 1.—Cyclic phosphoramide mustard derivatives constructed with the aid of various hydroxyamino acids



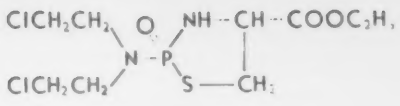
Derivative No.	R_1	R_2	Configuration
IA	H	OH	DL
IB	H	OH	L
IC	H	OH	D
II	C_6H_5	OH	*t*-DL
III	C_6H_5	OC_2H_5	*t*-DL
IV	C_6H_5	OC_2H_5	*e*-DL
V	$CH_2COOC_2H_5$	OC_2H_5	*t*-DL
VI	$C_6H_4NO_2$	OC_2H_5	*t*-DL

[5]Lapis K, and Szende B. Personal communication.

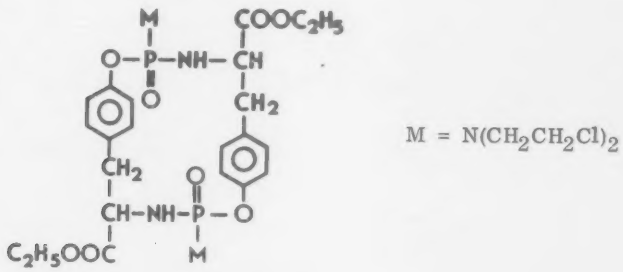
[6]Nemeth L, et al. Personal communication.



VII



VIII



IX

FIGURE 1.—Structures of cyclic phosphoramide mustards derived from various trifunctional amino acids.

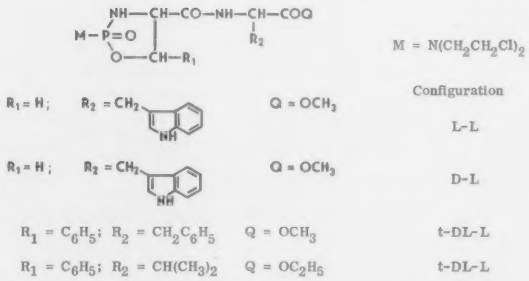

M = N(CH2CH2Cl)2
Configuration
R1 = H; R2 = CH2 Q = OCH3 L-L
R1 = H; R2 = CH2 Q = OCH3 D-L
R1 = C6H5; R2 = CH2C6H5 Q = OCH3 t-DL-L
R1 = C6H5; R2 = CH(CH3)2 Q = OC2H5 t-DL-L

FIGURE 2.—Peptide derivatives of cyclic phosphoramide mustards based on various hydroxyamino acids.

The stereoisomeric peptide derivatives based on L- and D-serine, containing an identical amino acid, L-tryptophan, as second component (table 4), did show a marked difference in toxicity although an identical degree of tumor inhibition was registered, but at different dose levels which corresponded to the LD10 values in each case. Therefore, the general rule that the D-amino acid-containing derivative has lower toxicity but decreased activity seems to be valid.

In the second group of peptides a more unusual trend can be observed: the D-D peptide (CB 40360/C) is not only less toxic but is also a more potent tumor inhibitor than the L-L derivative (CB 40360/B) (table 5). A similar behavior has been reported for aminochlorambucil, a derivative of an α-amino acid not known to occur in nature. In this case the D-form was also a more active tumor inhibitor than the L-form (13). Relative to this problem we should mention the stereochemical influence observed for compounds CB 40526 and CB 40527, which are the *erythro* and *threo* modifications respectively of an otherwise identical structure. The two compounds differ both in toxicity and in TI.

The peptide formation enables the introduction of a second amino acid side chain and a greater variation in the physicochemical parameters of the drug, such as hydrophobic aggregation forces at action, charge distribution in the molecule, and the spatial arrangements which play a role in the possible receptor binding or steric hindrance on the intramolecular level.

The influence of the second amino acid component is quite marked with regard to reduced toxicity. A good example of diminished toxicity is the comparison of compounds CB 40602 and CB 400120. The presence of triptophyl and valyl side chains seems to be especially beneficial.

An interesting theory has been put forward by Héléne (14) and Dimicoli and Héléne (15) with regard to the role of tryptophan in proteins. Because the indole ring may interact directly with bases in nucleic acids through stacking, interaction might be important in inducing local conformational changes which could introduce new hydrogen bonding possibilities for the recognition of bases by other amino acid side chains in proteins (or peptides).

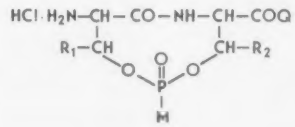
The side chain of valine, a nonpolar group, can increase lipid solubility of the molecule and can

TABLE 2.—Antitumor activity of cyclic phosphoramide mustard derivatives constructed with the aid of various hydroxyamino acids

Derivative No.	CB No.	ADJ/PC6A LD50 (mg/kg)	ADJ/PC6A ID90 (mg/kg)	ADJ/PC6A TI (LD50/ID90)	ADJ/PC5 % inhibition*	ADJ/PC5 TI	% inhibition and dose (mg/kg × days) in— Yoshida sc sarcoma	% inhibition and dose (mg/kg × days) in— NK/Ly ascites tumor
IA	40601	—	—	—	Inactive		75 (8 × 80), ref 6	—
IB†	40602	—	—	—	—	—	93 (8 × 80), ref 6	—
IC		—	—	—	—	—	99 (8 × 80), ref 6	—
II	40580	660	<400	>1.6	53	—	—	—
III	40527	950	350	2.7	—	—	—	—
IV	40526	1650	<250	>6.6	41	—		
V	40578	>1600	380	>4.2	96	1.5		
VI‡			—				Inactive	44 (8 × 10),§ footnote 5
IX	400121		Inactive				67 (10 × 25),§ footnote 5	60 (10 × 25),§ footnote 5

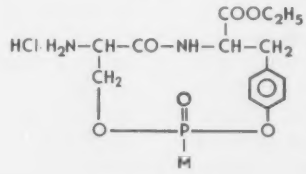
*Highest value of % inhibition in tumor system.
†Some activity against Walker 256 carcinosarcoma (LD50, 600 mg/kg).
‡48% inhibition against Ehrlich ascites sarcoma at an ip dose of 8 mg/kg × 10 days.
§Dose = LD10.

TABLE 3.—Mustard derivatives of phosphoramidic acid diesters obtained from hydroxyamino acid dipeptides



Derivative No.	R_1	R_2	Q	Configuration
XIVA	H	H	OH	DL-DL
XIVB	H	H	OH	L-L
XIVC	H	H	OH	D-D
XV	H	C_6H_5	OC_2H_5	DL-*t*-DL
XVI	C_6H_5	H	OH	*t*-DL-DL
XVII	C_6H_5	C_6H_5	OC_2H_5	*t*-DL-*t*-DL

XVIII



L-L

M = $N(CH_2CH_2Cl)_2$

TABLE 4.—Antitumor activity of peptides derived from amino acid based cyclic phosphoramide mustards

Derivative No.	CB No.	ADJ/PC6A LD50 (mg/kg)	ADJ/PC6A ID90 (mg/kg)	ADJ/PC6A TI (LD50/ID90)	% inhibition and dose (mg/kg × days) in— NK/Ly ascites tumor	% inhibition and dose (mg/kg × days) in— Yoshida sc sarcoma
X	400120	>1280	290	>4.4	35 (10 × 86),* footnote 5	17 (10 × 86), footnote 5
XI			—		.31 (10 × 260),* footnote 5	17 (10 × 260), footnote 5
XII			—		—	Inactive (9 × 50), footnote 5

*Dose = LD10 in mice.

TABLE 5.—Antitumor activity of compound XIII compared to the activity of CP in the same system*

Compound	Antitumor activity in— Walker 256 carcinosarcoma Dose (mg/kg × day)	% inhibition	% weight change in spleen	Ehrlich ascites sarcoma Dose (mg/kg × day)	T/C† × 100
XIII, molecular weight 494.4	7 × 40	88	−19	5 × 20	137
CP, molecular weight 230.1	7 × 20	90	−74	5 × 20	82

**From* footnote 6.
†T/C = treated/controls.

TABLE 6.—Phosphoramidic acid diester mustards obtained from 2 hydroxyamino acid-containing dipeptides*

Derivative No.	CB No.	Antitumor activity in— Walker 256 carcinosarcoma LD50 (mg/kg)	ID90 (mg/kg)	TI (LD50/ID90)	ADJ/PC6A LD50 (mg/kg)	ID90 (mg/kg)	TI (LD50/ID90)	Yoshida sc sarcoma Dose (mg/kg × day)	% inhibition
XIVA	40360/A	100	30	3.3		—		8 × 80	100†
XIVB	40360/B	60	—	Inactive		—			—
XIVC	40360/C	> 160	80	> 2		—			—
XV	400122		—		1125	170	6.6		—
XVII‡			—			—		10 × 15	49§
XVIII	400123		—		670	185	3.6		—

*As formulated in table 3.
†*From* ref 6.
‡Inactive against NK/Ly ascites tumor in mice at a dose level of 15 mg/kg × 10.
§*From* footnote 5.

especially enhance the formation of hydrophobic bonds to binding sites. A beneficial effect of the presence of valine with regard to toxicity has also been reported in the field of sarcolysin peptides by Larionov (8).

The last important point I would like to mention is that the second group of peptide derivatives (tables 3 and 6), which are not close analogs of CP because of their different ring system, also show marked tumor-inhibitory potential on a level quite comparable to the first group of peptides (fig 2, table 4) which are closer analogs of CP. Good examples are the compounds CB 400122 and CB 400120. Therefore, it can be concluded that peptide formation in this group is also suitable to influence therapeutic efficacy.

To summarize, there are indications that the relative intensities of antitumor activity, toxicity, and antitumor spectrum can be influenced by the following molecular manipulations: (*a*) variation of amino acid carrier; (*b*) extension of the chain length at least to a dipeptide; and (*c*) use of amino acids of different configuration. The pharmacokinetics, including bioactivation, seem to be markedly dependent upon the overall physicochemical properties and stereochemistry of the molecule in accordance with the recent interpretation of "chemical structure." According to disciplines of molecular pharmacology, the classic structural formulas symbolizing certain organic compounds should be interpreted as molecular structures, ie, as the complexes of spatial arrangements, the interrelations between the atoms in the molecule, the inherent charge distribution, and the mobility of these charges in the molecule.

The toxicity of cyclic phosphoramide mustard derivatives of amino acids and peptides was lower in quite a few cases (especially on a molar basis) than that of the parent compound CP, but the 90% tumor inhibition is exerted at relatively higher dose levels resulting in a much more narrow interval of active doses (as expressed by the lower TI values).

The combinations investigated so far seem to offer more variation of the spectrum of activity or a decrease of particular side effects than an overall improvement of therapeutic efficacy. Information gained by a detailed investigation of the exact nature of activation might render this group of compounds a useful tool in the elucidation of the mechanism of action of phosphoramide mustards.

Due to the presence of a number of substituents in the ring, hydrolytic activation might be preferred to oxidative activation (which is likely to be hindered). An additional argument in favor of this hypothesis is the finding of Arnold and Bourseaux (5) that the hydrolysis of the five-membered ring in B 485 is enhanced compared to the six-membered ring contained in B 578 (identical with CP). The latter compound has a significantly higher stability, particularly when enzymatic splitting is carried out with acidic phosphatase.

Further experiments are needed to establish the exact features of activation. Monitoring of hydrolytic decomposition in vitro and identification of breakdown products are currently receiving attention.

Macromolecular Carriers

The exploration of modifications of the free diffusion of the drug by carrier-mediated transport was extended to macromolecular carriers. Another approach in concentrating cytotoxic agents in tumor cells has been to use larger molecular weight carriers which can be taken up more selectively by tumor-cell endocytosis. De Duve et al (16) introduced the term "lysosomotropism" to designate the uptake of substances that enter lysosomes as part of an endocytizable complex (or conjugate) and are released in free form as a result of processing the carrier within the lysosomes.

The most interesting aspect of lysosomotropism is that it can be conferred artificially on almost any substance by suitable coupling with an appropriate carrier; such carriers include all major groups of macromolecules.

The combinations of macromolecules with CP or its analogs (which might be candidates for lysosomotropism) can be divided into three groups.

Based on the findings that some nitrogen mustard derivatives merely absorbed to protein could also be more selective than the original mustard alone (11,17), complexes of CP were formed with different proteins. In agreement with previous observations (18,19), it was found that CP was only marginally bound to proteins. Complexes formed with albumin (CB 3542) and fibrinogen (CB 3536) (17) had a drug content of 2.4% and 3.7% respectively. Biologic tests carried out on the ADJ/PC6A plasma-cell tumor indicated, for the fibrinogen complex only, some tendency for a decreased toxicity and increased therapeutic activity (when calculation was based on the actual drug content).

In the second group of experiments, covalent bonds were formed (20) by coupling *N,N*-bis(2-chloroethyl)phosphoramidic dichloride with fibrinogen (CB 3492), poly-DL-alanyl fibrinogen (CB 3495), and protamin (CB 3533). Two polypeptides, poly-DL-, L-, and D-serine (table 7) (2,21), and copoly-DL-seryl-L-glutamic acid were also investigated as carriers.

TABLE 7.—Structures of cyclic phosphoramide mustards derived from poly-serine

Derivative No.	Configuration
XIXA	DL
XIXB	L
XIXC	D

None of the protein combinations represented an outstanding improvement in therapeutic properties based on the screening data obtained in the ADJ/PC6A tumor system.

On the other hand, the poly-DL-serine derivative proved to be a potent tumor inhibitor, active not only against the Walker 256 carcinosarcoma, but also against the Yoshida sc sarcoma, Guérin carcinoma, and JKB ascites sarcoma (12). Slight growth retardation could be proved on benzpyrene-induced tumors. Screening data are summarized in table 8. The polypeptide combinations are insoluble in water and most organic solvents, but one of the carriers, poly-DL-serine, has a much better solubility than poly-L- and D-serine. This fact might offer an explanation for why both the poly-L- and D-serine mustard derivatives were found less active in the Walker 256 carcinosarcoma. The copoly-DL-seryl-L-glutamic acid derivative (serine:glutamine = 1:1) proved to be inactive against Ehrlich ascites and NK/Ly ascites tumors and had only marginal activity against Yoshida sc sarcoma. The polymer carrier might contain short polyserine sequences—being a random copolymer—but, seemingly, accumulation of active entities is not comparable to a homopolymer poly-serine chain.

The third experimental variation was realized by joining cyclic phosphoramide mustard derivatives with proteins. Compounds IA (CB 40601) and II (CB 40580) were chosen as suitable models, both derivatives having a free carboxyl group available. Coupling was achieved with the aid of a water-soluble carbodi-imide (1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-toluene-*p*-sulfonate) to human serum albumin. The conjugates contained about 3% drug, based on chlorine analysis and alkylating power, as measured by a modified Epstein technique (22). Screening of these compounds is currently in progress.

The basis for any improved selectivity achieved with macromolecular complexes and conjugates of drugs is not at all clear. Lysosomotropism is an attractive hypothesis; a lysosome-mediated effect must therefore be taken into consideration and examined experimentally. Once elucidated the mechanism may lead to more efficient use of the cytotoxic agents in the treatment of cancer.

REFERENCES

1. SZEKERKE M, CSÁSZÁR J, and BRUCKNER V. Serine peptides as carriers of cytoactive groups. Chem Ind, 1964, pp 1385-1386.
2. SZEKERKE M, CSÁSZÁR J, and BRUCKNER V. Cytotoxische Gruppen enthaltende Serin-, Seryl-serin- und Polyserin-Derivate. Acta Chim Acad Sci Hung 46:379-390, 1965.
3. SZEKERKE M, KAJTÁR MT, and BRUCKNER V. Synthese cyclischer *N*-Lost-Derivate aus β-substituierten Serinen, Cystein und Lysin. Acta Chim Acad Sci Hung 47:231, 1966.
4. SZEKERKE M. New cyclophosphamide analogues derived from hydroxyamino acids and some peptides thereof. Ann Univ Sci Budapestinensis Sect Chim 13:57-67, 1972.
5. ARNOLD H, and BOURSEAUX F. Synthese und Abbau cytostatisch wirksamer cyclischer *N*-Phosphamidester des Bis-(β-chloräthyl)-amins. Angew Chem 70:539-544, 1958.
6. SZEKERKE M. Citoaktiv csoportokat tartalmazó peptidszármazékok. Kémiai Közlemények. Hung Acad Sci 28:235-250, 1967.
7. BERGEL F, STOCK JA, and WADE R. Peptides and macromolecules as carriers of cytotoxic groups. *In* Biological Approaches to Cancer Chemotherapy (Harris RJC, ed). New York, Academic Press, 1961, pp 125-134.

TABLE 8.—Antitumor activity of cyclic phosphoramide mustard derivatives of poly-DL-, L-, and D-serine

Derivative No.	CB No.	Antitumor activity in— Walker 256 carcinosarcoma LD50 (mg/kg)	ID90 (mg/kg)	TI (LD50/ID90)	Sarcoma 180 Dose (mg/kg × day)	C/T × 100
XIXA*,†	40353/A	240	40	6	10 × 50	155
XIXB	40353/C	> 400		Inactive	—	—
XIXC	40353/D	> 400	180	> 2.2	—	—

**From* ref 12: data for activity against Yoshida ascites sarcoma, Guérin carcinoma, and JKB ascites sarcoma.
†Activity of 100-mg ip single dose against Furth leukemia in rat; T/C = 1.28.

8. Larionov LF. An approach to the creation of antitumour drugs with diverse action spectra. *In* Biological Approaches to Cancer Chemotherapy (Harris RJC, ed). New York, Academic Press, 1961, pp 139-148.
9. Szekerke M, Wade R, and Bergel F. Cyto-active amino-acids and peptides. XI. Nitrogen mustard derivatives of glutamic acid and related compounds. J Chem Soc, 1965, pp 1907-1912.
10. Rosenoer VM, Mitchley BCV, Roe FJC, et al. Walker carcinosarcoma 256 in study of anticancer agents. I. Method for simultaneous assessment of therapeutic value and toxicity. Cancer Res (Suppl) 26:937-941, 1966.
11. Wade R, Whisson ME, and Szekerke M. Some serum protein nitrogen mustard complexes with high chemotherapeutic selectivity. Nature 215:1303-1304, 1967.
12. Szende B, and Németh K. Effekt von Serin-Derivaten mit zytostatischen Gruppen auf das Wachstum transplantabler Tiergeschwülste. Naturwissenschaften 52:63-64, 1965.
13. Bergel F, Lewis GE, Orr SFD, et al. Interaction between carbonyl groups and biologically essential substituents. I. The effect of ketones on optically active amino-derivatives. J Chem Soc, 1959, pp 1431-1437.
14. Hélène C. Role of aromatic amino-acid residues in the binding of enzymes and proteins to nucleic acids. Nature [New Biol] 234:120-121, 1971.
15. Dimicoli JL, and Hélène C. Interactions of aromatic residues of proteins with nucleic acids. I. Proton magnetic resonance studies of the binding of tryptophan-containing peptides to poly(adenylic acid) and deoxyribonucleic acid. Biochemistry 13:714-723, 1974.
16. De Duve C, De Barsy T, Poole B, et al. Commentary: Lysosomotropic agents. Biochem Pharmacol 23:2495-2531, 1974.
17. Szekerke M, Wade R, and Whisson ME. The use of macromolecules as carriers of cytotoxic groups (part II) nitrogen mustard—protein complexes. Neoplasma 19:211-215, 1972.
18. Sanderson RP, McKenna JM, and Blakemore WS. Cytotoxic activity compared with photometric measurements of selected alkylating agents. Nature 205:479-481, 1965.
19. Hill BT, and Harrap KR. Modification of the alkylating ability of cyclophosphamide by fresh plasma. Chem Biol Interact 5:117-125, 1972.
20. Szekerke M, Wade R, and Whisson ME. The use of macromolecules as carriers of cytotoxic groups (part I) conjugates of nitrogen mustards with proteins, polypeptidyl proteins and polypeptides. Neoplasma 19:199-209, 1972.
21. Szekerke M. Syntheses of poly-DL-serine derivatives containing cytotoxic groups. Nature 199:280, 1963.
22. Hopwood WJ, and Stock JA. The effect of macromolecules upon the rates of hydrolysis of aromatic nitrogen mustard derivatives. Chem Biol Interact 4:31-39, 1971.

Deactivation of Cyclophosphamide (NSC-26271) Metabolites by Sulfhydryl Compounds[1,2]

U. Draeger,* G. Peter, and H.-J. Hohorst[3]

SUMMARY

The reaction of 4-hydroxycyclophosphamide (4-hydroxy-CP) with sulfhydryl (SH) compounds was studied, and although the cytotoxic activity was lost a significant stabilization of the alkylating capacity was observed at the same time. We were able to show that the reaction of 4-hydroxy-CP with thiols leads to an equilibrium between the reaction product and the starting substrates. On the basis of this equilibrium the increased stabilization of the alkylating capacity of the 4-hydroxy-CP derivatives by raising the SH concentration can be explained. The different degrees of stabilization depending on the structure of the thiol results from different equilibria. The effect on the toxification reaction resulting from this equilibrium, in connection with the tautomeric equilibrium between 4-hydroxy-CP and aldophosphamide, is discussed.

[Cancer Treat Rep 60:355-359, 1976]

It was previously observed (1) that the cytotoxic activity of urine collected from rats after cyclophosphamide (CP) treatment could be reduced by cysteine in weak alkaline aqueous solution without affecting the alkylating capacity of the sample. Since the cytotoxic activity of the urine was mainly based on the presence of the primary activated CP metabolite (2), the loss of the cytotoxic activity as tested in Yoshida ascites tumor cells may have resulted from cysteine affecting the permeation of the activated metabolite 4-hydroxycyclophosphamide (4-hydroxy-CP) into the cell or preventing the alkylation within the cell, or otherwise may have been based on a direct reaction of cysteine and 4-hydroxy-CP while the β-chloroethyl groups remained unchanged. These considerations led to a study of the reactions between 4-hydroxy-CP and sulfhydryl (SH) compounds.

MATERIALS AND METHODS

^{3}H-CP (specific activity, approximately 180 microcuries/μmol), uniformly labeled at the β-chloroethyl groups, was obtained from Prof. Schaumlöffel (Klinik und Polyklinik für Nuclearmedizin, Marburg, West Germany). 4-Hydroxy-CP was synthesized according to Takamizawa et al (3) and Peter et al (4). ^{3}H-4-hydroxy-CP (specific activity, 0.1 microcurie/μmol), uniformly labeled at the β-chloroethyl groups, was produced from the peroxide by the same method. The purity was chromatographically proved according to a previously described method (5). L(+)-Cysteine, *N*-acetyl-L-cysteine, and the cellulose plates for the thin-layer chromatography studies were supplied by E. Merck AG, Darmstadt, West Germany. D,L-(3-^{14}C)-cysteine hydrochloride (^{14}C-cysteine; specific activity, 50 microcuries/μmol) was supplied by Amersham Buchler GmbH, Wenden, West Germany. Reduced crystalline glutathione was supplied by Boehringer Mannheim GmbH, Mannheim, West Germany. 4-(4-Nitrobenzyl)pyridine (NBP) was supplied by EAG-Chemie, Steinbach, West Germany. 5,5-Dithio-bis-(2-nitrobenzoic acid) (DTNB) was supplied by Calbiochem, Los Angeles, California. 2-(4-*t*-Butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (butyl-PBD) was supplied by W. Zinsser, Frankfurt, West Germany.

[1]Supported by Bundesministerium für Forschung und Technologie.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany.

*Reprint requests to: Dr. U. Lenssen, Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, Theodor-Stern-Kai 7, 6000 Frankfurt 70, West Germany.

NBP test.—The total concentration of the alkylating and potential alkylating compounds (defined as the alkylating capacity) in solution was determined according to Friedman and Boger (6) after previous acid hydrolysis in 1 N HCl at 100°C for 10 minutes. All alkylating and potential alkylating compounds were determined by this method.

NBP test for the demonstration of alkylating material by thin-layer chromatography.—The cellulose plates were sprayed with 0.025 N acetate buffer (pH 4.6) and then with a solution of 2.5% NBP in acetone. The plates were heated for 10 minutes at 150°C and after cooling to room temperature they were sprayed with 0.3 N NaOH. Alkylating substances appeared as blue stains.

DTNB test.—SH groups were determined according to Ellman (7).

Iodine-azide test.—This test was used for the detection of sulfur-containing compounds on cellulose plates. The test gives a positive reaction to sulfur-containing amino acids, sulfides, and thioethers (8,9).

Measurement of radioactivity.—The radioactivity of solutions was determined in the liquid scintillation counter (PW 4510/01; Philips, Eindhoven, The Netherlands) by adding 5-100 μl of the radioactive solution to 12 ml of scintillation liquid. The scintillation liquid contained 6 g of butyl-PBD in 600 ml toluene and 400 ml methylglycol. The radioactivity of compounds separated on cellulose plates by electrophoresis was measured in the scintillation counter after scraping the cellulose into the vial, adding the scintillation liquid, and mixing vigorously.

Thin-layer electrophoresis (TLE).—The cellulose plates were impregnated with a buffer (pH 6.5) of water:pyridine:glacial acetic acid (89:10:1). The apparatus used was the Original Frankfurt Type Mini 65 made by Hormuth and Vetter, Wiesbaden, West Germany (field strength = 27 volts/cm; temperature = 2°C; running time = 1.5 hours).

RESULTS AND DISCUSSION

We first studied the above-mentioned effect of cysteine added to urine containing the active metabolite 4-hydroxy-CP. The result was a loss of the cytotoxic activity without an effect on the alkylating capacity. 4-Hydroxy-CP was incubated with different SH compounds in equimolar concentrations in 0.07 M phosphate buffer (pH 7) at 37°C. The decrease of the SH groups was measured with the DTNB test and is shown in figure 1, demonstrating a direct reaction of the thiol with 4-hydroxy-CP.

In the measurement of the alkylating capacity, when 4-hydroxy-CP was incubated with cysteine,

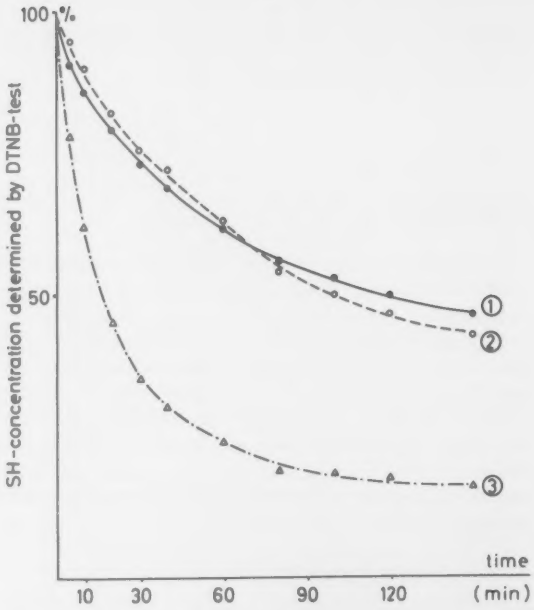


FIGURE 1.—Decrease of titratable SH groups during the incubation of 4-hydroxy-CP with 10 mM *N*-acetyl-L-cysteine (1), glutathione (2), or cysteine (3) in 0.07 M phosphate buffer (pH 7) at 37°C. Aliquots were taken at different times and the SH concentration was determined by the DTNB test.

glutathione, and *N*-acetyl-L-cysteine under the same conditions, no decrease or only a small decrease could be observed in the first hour (fig 2), while the control, 4-hydroxy-CP without SH compounds, showed the typical loss of the alkylating capacity resulting from hydrolysis. It is therefore possible to reduce the alkylation of water or SH groups by 4-hydroxy-CP under these conditions, or, in other words, the alkylating capacity of 4-hydroxy-CP can be stabilized in the presence of SH compounds while the cytotoxic activity is simultaneously lost.

Figure 2 also shows a stabilization of the alkylating capacity depending on the concentration of the thiol, in such a manner that a doubling of the relative SH concentration leads to a stronger stabilization of the alkylating capacity (lower part of fig 2). Under physiologic conditions of pH and temperature the alkylating capacity of 4-hydroxy-CP can be nearly completely stabilized with the double concentration of SH compounds up to 10-15 hours, while the cytotoxic activity is lost at the same time.

This surprising phenomenon, as a reduction of the alkylating activity of 4-hydroxy-CP by SH

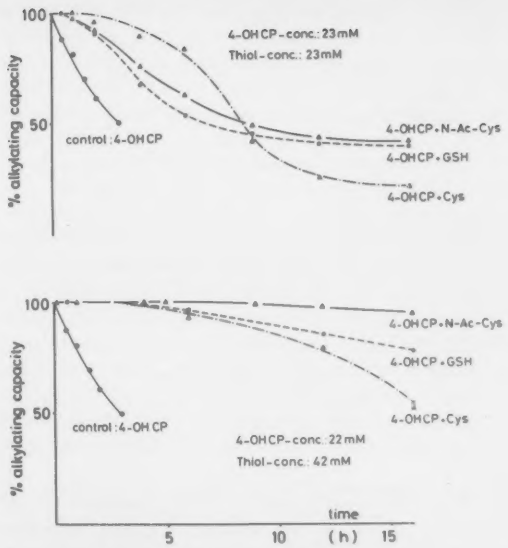


FIGURE 2.—Alkylating capacity of 4-hydroxy-CP (23 mM) after incubation with the different thiols (23 mM, upper part of the fig, or 42 mM, lower part of the fig) in 0.07 M phosphate buffer (pH 7) at 37°C. Aliquots were taken at different times and the alkylating capacity was determined by the NBP test.

compounds, leads to the following conclusions: (*a*) 4-hydroxy-CP itself is not an alkylating substance (10); (*b*) the reaction does not take place at the β-chloroethyl groups but at the oxazaphosphorine ring of 4-hydroxy-CP; (*c*) the reaction evidently reduces or prevents the release of the real alkylating agent.

Hence, the question arises, which reaction takes place between the SH compound and the oxazaphosphorine ring. The most likely candidate is the crypto-aldehyde of 4-hydroxy-CP or the aldehyde group of the tautomeric aldophosphamide. To clarify the alternative possibilities, the stoichiometry of the thiol/4-hydroxy-CP reaction was studied. The best way was to isolate the reaction products. Unfortunately, direct isolation and analysis were not possible because the stability of the products was insufficient. Another possibility was to use radioactive substrates for the reaction and to separate the reaction product from the starting substrates under mild conditions. The best way to achieve separation was by TLE as shown in figure 3. The distance between the products and the reactants after electrophoresis was sufficient. Moreover, the figure shows that 4-hydroxy-CP does not react quantitatively with the thiol incubated in equimolar concentrations since unreacted 4-hydroxy-CP remains. This observation may indicate an equilibrium.

Nevertheless, this method provides an opportunity to study the stoichiometry of the reaction products. ^{3}H-4-hydroxy-CP (specific activity, 0.1 microcurie/μmol) was incubated with ^{14}C-cysteine (specific activity, 0.25 microcurie/μmol) in 0.07 M phosphate buffer (pH 7) at 37°C for 1 hour. The reaction product was separated by TLE and the ^{3}H and ^{14}C radioactivities were measured in the liquid scintillation counter. The specific ^{3}H and ^{14}C activities of the reaction product were identical compared with those of the starting substrates. This means that 4-hydroxy-CP reacts only with one thiol and consequently this excludes a mercaptalization of aldophosphamide and points to a reaction of 4-hydroxy-CP itself. This and the nature of the reaction product could be proved by synthesis of analog compounds as well as by infrared, proton magnetic resonance (4), and field desorption mass spectroscopy (11).

The incomplete reaction between the activated CP metabolite and SH compounds in equimolar concentrations as demonstrated in figure 3, as well

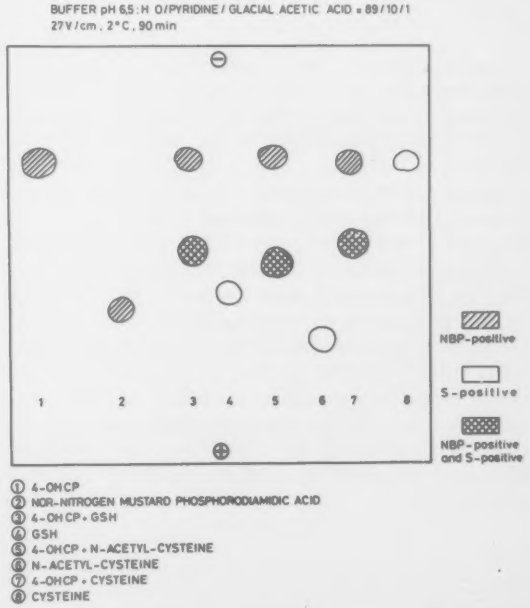


FIGURE 3.—Electrophoretic separation of the products after reaction of 4-hydroxy-CP. 4-Hydroxy-CP (concentration, 50 mM) was incubated with the SH compounds (concentration, 50 mM) in 0.07 M phosphate buffer (pH 7) at 37°C for 2 hrs. Two μl of the solutions was separated on a cellulose plate by TLE (*see* Materials and Methods). 1 = 4-hydroxy-CP; 2 = *nor*-nitrogen mustard phosphorodiamidic acid; 3 = 4-hydroxy-CP + glutathione; 4 = glutathione; 5 = 4-hydroxy-CP + *N*-acetyl-L-cysteine; 6 = *N*-acetyl-L-cysteine; 7 = 4-hydroxy-CP + cysteine; 8 = cysteine.

as the obviously greater stabilization of the alkylating capacity by raising the thiol concentration (fig 2), points to an equilibrium between the reaction product, 4-hydroxy-CP, and the thiol. This could be proved by an exchange reaction of cysteine and ^{14}C-labeled cysteine. A total of 4 μmols of ^{3}H-4-hydroxy-CP (specific activity, 0.1 microcurie/μmol) was incubated with the same quantity of cysteine in 100 μl of 0.07 M phosphate buffer (pH 7) at 37°C for 1 hour. An aliquot was taken and the reaction product was separated by TLE. Then, 0.1 microcurie of high-activity ^{14}C-cysteine (specific activity, 50 microcuries/μmol) was added, so that the final cysteine concentration was nearly unchanged. The sample was incubated for an additional 30 and 60 minutes at 37°C and the reaction product was separated again by TLE. The ^{3}H and ^{14}C radioactivities were measured and the results are given in table 1.

The exchange of the unlabeled cysteine moiety of the 4-hydroxy-CP/cysteine product by ^{14}C-cysteine was relatively fast and was complete within 30 minutes at 37°C. This proves that an equilibrium is formed between the 4-hydroxy-CP/thiol product and the starting substrates.

The dependence of the stabilization of the alkylating capacity, or of the reduction of the alkylating rate, on the concentration of the SH compound (fig 2) is understandable on the basis of such an equilibrium. Moreover, the different stabilization of the alkylating capacity, which depends on the structure of the thiol component, as can also be observed in the upper part of figure 2, may result in different positions of the equilibria. To study this, ^{3}H-4-hydroxy-CP was incubated with equimolar concentrations of cysteine, glutathione, and *N*-acetyl-L-cysteine respectively in 0.07 M phosphate buffer (pH 7) at 37°C. A total of 10 μl of the incubation mixture was taken at different times, applied in a line on the buffer-impregnated cellulose plate, and separated by TLE. The whole radioactivity on the plate was taken as 100% and the quantity of the 4-hydroxy-CP/thiol product was calculated relative to this. The results are shown in figure 4. The reaction rate, as well as the maximum conversion, depends on the structure of the SH compound. The reaction rate of 4-hydroxy-CP and cysteine is the fastest when compared with that of 4-hydroxy-CP and glutathione or *N*-acetyl-L-cysteine respectively, which show the same reaction rates. The greatest maximum conversion was obtained with cysteine (nearly 90%), while the other thiols both react with 4-hydroxy-CP to a maximum conversion of 50%. This allows an estimation of the equilibria. The 4-hydroxy-CP/cysteine equilibrium lies nearly completely on the product side, whereas the other equilibria lie approximately at the same concentrations of the product and the starting substrates. This again explains the different influence of the different SH compounds at the same concentrations on the alkylating capacity (fig 2).

As described previously (5,10) the release of the alkylating agent during the toxification reaction limits the rate of the spontaneous breakdown of 4-hydroxy-CP under physiologic conditions. Moreover, the reaction is first order with respect to the starting substrate 4-hydroxy-CP or aldophosphamide. Since the concentration of aldophosphamide is established by the 4-hydroxy-CP/thiol equilibrium in connection with the tautomerism equilibrium, the reaction rate of the release of the alkylating agent must be inversely proportional to the concentration of the free SH compound. The thiol/4-hydroxy-CP equilibrium in connection with tauto-

TABLE 1.—Radioactivity of the reaction product

Radioactivity measured—	^{3}H activity (dpm)	^{14}C activity (dpm)
1 hr before adding ^{14}C-cysteine (37°C)	44,600	0
0.5 hr after adding ^{14}C-cysteine (37°C)	44,150	109,450
1 hr after adding ^{14}C-cysteine (37°C)	44,500	109,725

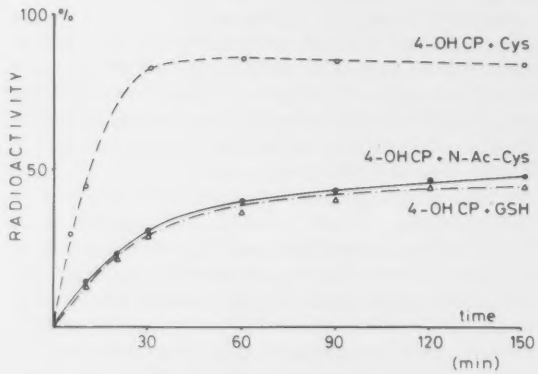
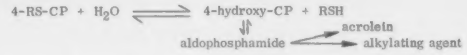


FIGURE 4.—Formation of the reaction products during the incubation of 4-hydroxy-CP with different SH compounds. ^{3}H-4-hydroxy-CP (concentration, 50 mM) was incubated with the SH compounds (concentration, 50 mM) in 0.07 M phosphate buffer (pH 7) at 37°C. Aliquots were taken at different times and separated on cellulose plates by TLE (see Materials and Methods). The whole radioactivity of an aliquot was set as 100% and the amount of the product was calculated relative to this.

meric equilibrium and the toxification reaction is stated as



The equilibrium constant of the equilibria are

$$\frac{[\text{4-hydroxy-CP}] \cdot [\text{RSH}]}{[\text{4-RS-CP}] \cdot [\text{H}_2\text{O}]} = K_S \qquad \frac{[\text{4-hydroxy-CP}]}{[\text{aldophosphamide}]} = K_T$$

Connecting both terms, the following equation results, which shows that the aldophosphamide concentration is inversely proportional to the SH concentration

$$\frac{[\text{aldophosphamide}] \cdot K_T \cdot [\text{RSH}]}{[\text{4-RS-CP}] \cdot [\text{H}_2\text{O}]} = K_S$$

$$[\text{aldophosphamide}] = K' \frac{[\text{4-RS-CP}]}{[\text{RSH}]}$$

The toxification reaction rate is defined as

$$v_{\text{Toxification}} = k_T \cdot [\text{aldophosphamide}]$$

When the aldophosphamide concentration is replaced by the above term, the toxification rate is inversely proportional to the SH concentration

$$v_{\text{Toxification}} = k_T \cdot K' \frac{[\text{4-RS-CP}]}{[\text{RSH}]}$$

This alteration of the alkylation rate by SH compounds, determined as the decrease of the alkylating capacity of 4-hydroxy-CP, is illustrated in figure 2. It is the most evident demonstration of the fact that the activated CP metabolites of this kind, and those that have not passed the toxification reaction, are not alkylating compounds (10).

REFERENCES

1. Primack A. Amelioration of cyclophosphamide-induced cystitis. J Natl Cancer Inst 47:223-227, 1971.
2. Hohorst H-J, Ziemann A, and Brock N. Alkylierende Substanzen in Serum und Urin nach Injektion von Cyclophosphamid. Arzneim Forsch 15:432-434, 1965.
3. Takamizawa A, Matsumoto S, Iwata T, et al. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
4. Peter G, Wagner T, and Hohorst H-J. Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. Cancer Treat Rep 60:429-435, 1976.
5. Voelcker G, Draeger U, Peter G, et al. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin-layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
6. Friedman OM, and Boger E. Colorimetric estimation of nitrogen mustard in aqueous media. Anal Chem 33:906-910, 1961.
7. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-77, 1959.
8. Chargaff E, Levine C, and Green C. Techniques for the demonstration by chromatography of nitrogenous lipide constituents, sulfur-containing amino acids, and reducing sugars. J Biol Chem 175:67-71, 1948.
9. Awe W, Reinecke J, and Thum J. Die Anwendung der Jod-Azid-Reaktion im Papierchromatogramm. Naturwissenschaften 41:528, 1954.
10. Hohorst H-J, Draeger U, Peter G, et al. The problem of oncostatic specificity of cyclophosphamide (NSC-26271): studies on reactions that control the alkylating and cytotoxic activity. Cancer Treat Rep 60:309-315, 1976.
11. Przybylski M, Ringsdorf H, Voelcker G, et al. Characterization of cyclophosphamide (NSC-26271) metabolites and related derivatives by field desorption and electron-impact mass spectrometry. Cancer Treat Rep 60:509-516, 1976.

Stereochemistry, Metabolism, and Antitumor Activity of 4-Hydroperoxyisophosphamide (NSC-227114) and its Stereoisomer

A. Takamizawa,* T. Iwata, K. Yamaguchi, O. Shiratori, M. Harada, Y. Tochino, and S. Matsumoto[1,2]

SUMMARY

An acid-catalyzed isomerization of 4-hydroperoxyisophosphamide, a synthetic compound with potential antitumor activity, produced a new crystalline epimer having an inverted stereochemistry at the phosphorus atom. The stereochemistry of these epimeric hydroperoxides is discussed on the basis of their chemical and nuclear magnetic resonance properties. The epimers show essentially the same metabolic behavior. This behavior is, however, different from that of isophosphamide (IP) which is metabolized in part via pathways ineffective for its activation. The newly isolated epimer shows slightly higher activity against some kinds of experimental tumors than the original hydroperoxide, suggesting that the inverted stereochemistry of the alkylating group at the phosphorus atom is effective in promoting the antitumor activity of the activated species of IP.

[Cancer Treat Rep 60:361-368, 1976]

There is considerable evidence that the first step of the in vivo activation of cyclophosphamide (CP) (*1*) involves C_4-hydroxylation of its 1,3,2-oxazaphosphorinane ring (1). Previously, we have reported that the chemically synthesized 4-hydroxycyclophosphamide (4-hydroxy-CP) (*2*), as well as 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP) (*3*), showed pronounced cytostatic activity in both in vivo and in vitro experiments, providing confirmatory evidence that C_4-hydroxylation is indeed responsible for the activation of CP (2). We have also reported that the similar C_4-functionalization of isophosphamide (IP) resulted in marked activation of the drug (3).

The stereochemical aspects of the C_4-oxygen functionality and the alkylating groups in activated CP and IP derivatives are a matter of considerable significance with respect to the structure-activity relationships of these antitumor agents. The most recent X-ray work by Camerman et al has revealed that the 4-hydroperoxy-CP prepared by us has a chair conformation in which the C_4-OOH group is *axial* and *cis* to the phosphoryl oxygen (4).

The molecular conformation of 4-hydroperoxy-CP can be stabilized by intramolecular interaction between the C_4-OOH and P = O groups, this presumably being a major reason for the stereoselective formation of a product having such a stereochemistry. A quite similar stereochemistry has also been proposed for the 4-hydroxycyclophosphamide anhydro-dimer (5) (4-peroxycyclophosphamide [6]) (*4*), which is directly derivable from CP by Fenton oxidation (7) or from 4-hydroperoxy-CP by partial reduction (5,6).

In the course of studies on the chemistry of C_4-functionalized 1,3,2-oxazaphosphorinane derivatives, we have found that 4-hydroperoxyisophosphamide (4-hydroperoxy-IP) can readily be converted into a crystalline stereoisomer which also shows high antitumor activity against some kinds of experimental tumors. We now report these results, together with the results of comparative metabolic studies on the two epimeric IP derivatives (fig 1).

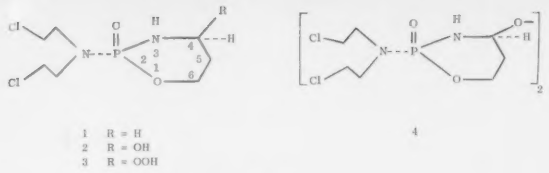
Stereochemistry

Due to the chirality at the phosphorus and C_4-carbon atoms, two epimers can theoretically exist for the C_4-functionalized 1,3,2-oxazaphospho-

[1]Shionogi Research Laboratory, Shionogi and Co, Ltd, Osaka, Japan.

[2]The authors wish to thank Drs. K. Tori and Y. Terui for a number of valuable discussions on the nuclear magnetic resonance data, and Dr. Y. Matsui and Mr. T. Takasuka for discussions and measurements of the infrared spectra. We also thank I. Makino, H. Kasai, N. Uchida, and S. Takase for their technical assistance.

*Reprint requests to: Dr. A. Takamizawa, Shionogi and Co, Ltd, Fukushima-ku, Osaka, 553, Japan.



1 R = H
2 R = OH
3 R = OOH

4

FIGURE 1

rinanes, including the activated CP and IP derivatives. However, until now there has been no report on the isolation of such stereoisomers, although some examples have been recorded for 1,3,2-dioxaphosphorinanes (8-13).

We have found that 4-hydroperoxy-IP (***5a***), which was obtained by ozonolysis of an appropriate olefin, readily isomerizes in the presence of an acid catalyst to an epimer as follows. In the presence of a catalytic amount of *p*-toluenesulfonic acid in chloroform at room temperature, ***5a*** gave a 1:1 equilibrium mixture with an isomer ***5b***. By column chromatography on silica gel-acetone:chloroform (1:2), ***5b*** could be isolated in a crystalline state as a faster migrating component: colorless prisms from acetone-ether (1:5), melting point (mp) 75°C-76°C (dec 112°C). Elemental analysis and molecular weight measurements in methanol agreed with the formula $C_7H_{15}N_2O_4PCl_2$. The chemical properties of the isomer ***5b*** are compared with those of ***5a*** (fig 2). Both ***5a*** and ***5b*** quantitatively produced 4-ketoisophosphamide (4-keto-IP) (***6***) (mp 117°C-118°C) under the action of ferrous sulfate in aqueous chloroform, while treatment with triethylphosphite in dichloromethane converted them into the corresponding 4-hydroxyisophosphamides (4-hydroxy-IP) ***7a*** (mp 74°C-75°C) and ***7b*** (mp 49°C-50°C) in good yield. Treatment of ***5a*** and ***5b*** with aqueous alkali (1 N KOH) in chloroform yielded the corresponding bicyclic peroxides [6-(2-chloroethylamino)perhydro[1,2,4]dioxazino[4,3-c][1,3,2]oxazaphosphorine-6-oxides] ***8a*** (mp 127°C-129°C) and ***8b*** (mp 103°C-105°C), which were also found to be in equilibrium in the presence of *p*-toluenesulfonic acid in chloroform at room temperature (***8a/8b*** = 4/5). All these epimeric IP derivatives were obtained in a stereochemically pure state. The epimer of 4-hydroperoxy-IP (***5b***) was also found to be obtainable in a low yield (5%) by the ozonolysis of *O*-(3-butenyl)-*N,N'*-bis(2-chloroethyl)phosphorodiamidate (***9***), the product being accompanied by 4-keto-IP (***6***) (5%) and a trace amount of a new compound ***10*** (mp 98°C-99°C) after repeated column chromatography with silica gel-acetone:chloroform (1:2) of the mother liquor of the major product ***5a*** (30%-35%). In the case of the synthesis of 4-hydroperoxy-CP (***3***), however, no epimeric product could be isolated, and attempted acid-catalyzed isomerization of ***3*** resulted in the formation of the dimeric peroxide ***4*** in a 40% yield (fig 3).

The stereochemistry of the 1,3,2-dioxaphosphorinanes has been extensively studied by nuclear magnetic resonance (nmr) spectroscopy, in which the P-O-C-H vicinal coupling constant is used as a useful parameter for assignment of their preferred conformation since the coupling is known to be greatly dependent on the corresponding dihedral angle (14-17). Similar angular dependence of P-N-C-H coupling has also been applied to the stereochemical assignment of the 1,3,2-oxazaphospholidines (18). Thus the nmr data of the C_4-proton of C_4-functionalized 1,3,2-oxazaphosphorinanes are also considered to be useful for prediction of their stereochemistry.

In table 1, the C_4-proton nmr data of the IP derivatives are listed and compared with those of CP derivatives.

The J(P-N-C_4-H) values of the epimeric 4-hydroperoxy-CP (***5a,5b***), given in table 1, are consistent with the reported P-O-C-H (*equatorial*) coupling

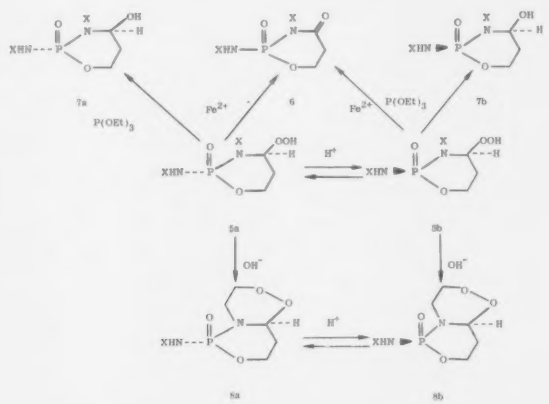


FIGURE 2.—X = CH_2CH_2Cl.

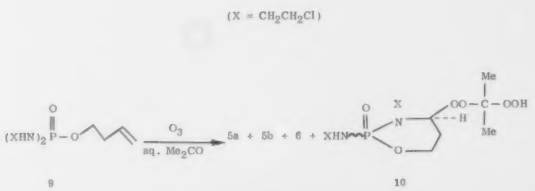


FIGURE 3

TABLE 1.—60 MHz nmr data for the C_4-proton of C_4-functionalized IP and CP derivatives

Compound	Solvent	$\delta(C_4\text{-H})$ (ppm)	Appearance	$J(P\text{-}N\text{-}C_4\text{-H})$ (Hz)	$\Sigma J(C_4\text{-H}, C_5\text{-H})$ (Hz)
5a	d_6-DMSO	4.96	d of t*	19.5	6.0
5b	d_6-DMSO	5.02	d of t	18.0	8.1
	$CDCl_3$	5.09	d of t	20.3	7.0
7a	D_2O	5.05	d of t	18.0	7.0
7b	D_2O	5.08	d of t	18.4	7.2
	$CDCl_3$	4.97	d of t	21.6	6.8
8a	$CDCl_3$	5.38	d of dd	22.5	7.0
	d_6-DMSO	5.34	d of t*	18.6	9.3
8b	$CDCl_3$	5.48	d of dd	5.7	14.0
	d_6-DMSO	5.36	d of dd*	5.0	15.5
10	$CDCl_3$	5.18	d of t	21.0	7.4
3	d_6-DMSO	4.96	d of t*	24.5	7.2
4	d_6-DMSO	5.22	d of t*	28.0	7.0
N-Methyl-4-hydroperoxy-CP (***14***)	d_6-DMSO	4.82	d of t	20.6	6.0
N-(*n*-Propyl)-4-hydroperoxy-CP (***15***)	d_6-DMSO	4.92	d of t	21.4	5.0

*After addition of D_2O.

constants of 2-dialkylamino-1,3,2-dioxaphosphorinane-2-oxides with a rigid chair conformation (17). This indicates that both of the epimers have an equatorial C_4-proton, which is also predictable from the small couplings between C_4- and C_5-protons (*see* $\Sigma J(C_4\text{-H}, C_5\text{-H})$ values in table 1). The nmr data of the IP derivatives listed in table 1 were found to be temperature-independent within the range −53°C-72°C, indicating that the six-membered ring of these compounds has a stable chair conformation. The nmr results, therefore, suggest that the isomerization of ***5a*** proceeds with retention of the C_4-configuration, and we believe that it must involve a stereomutation of the $P\text{-}NHCH_2\text{-}CH_2Cl$ group from *equatorial* to *axial*. A similar type of acid-catalyzed stereomutation at the phosphorus atom has been reported for 2-dimethylamino-4,6-dimethyl-1,3,2-dioxaphosphorinane-2-oxide (***11***), which also isomerized to an epimer ***12*** with inversion of the $P\text{-}N(CH_2)$ group from *equatorial* to *axial* (11) (fig 4).

The tentatively assigned structures of the two epimeric 4-hydroperoxy-IPs ***5a*** and ***5b*** can account for their different properties as follows. ***5b*** shows

11 12

FIGURE 4

a greater solubility in water than ***5a*** (***5a*** = 4 mg/ml, ***5b*** = 25 mg/ml at room temperature), suggesting that the polar groups (C_4-OOH and P = O) in ***5a*** are masked by an intramolecular hydrogen bond, while such interaction is impossible in ***5b***. The molecular-weight measurement of ***5b*** in a solvent of low polarity such as chloroform gave values intermediate between those for monomer and dimer, suggesting a partial molecular association, while a value corresponding to a monomeric form was obtained for ***5a***. Comparative infrared studies of ***5a*** and ***5b*** supported the suggested intramolecular hydrogen bonding in ***5a*** and its absence in ***5b***.[3] The assigned molecular structures of ***5a*** and ***5b*** are consistent with recent X-ray studies by Camerman,[4] confirming the *cis-diaxial* stereochemistry of P = O and C_4-OOH groups in ***5a*** and the P = O *equatorial*, C_4-OOH *axial* stereochemistry in ***5b*** (fig 5).

As is apparent in table 1, the nmr data of ***8b*** are greatly different from those of other IP derivatives. The smaller $J(P\text{-}N\text{-}C_4\text{-H})$ value, as well as the greater $\Sigma J(C_4\text{-H}, C_5\text{-H})$ value, is indicative of an *axial* configuration of the C_4-proton in ***8b***. We believe that the formation of ***8b*** with different stereochemistry at C_4 is best rationalized by assuming a common intermediate (***13***), which is sterically unfavored and turns into a more stable conformer ***8b***, both in the acid-catalyzed isomerization of ***8a*** and the alkali treatment of ***5b*** (fig 6).

[3]Takasuka T, and Matsui Y. Unpublished results.

[4]The authors are most grateful to Dr. A. Camerman and coworkers for making the X-ray analysis data available before publication.

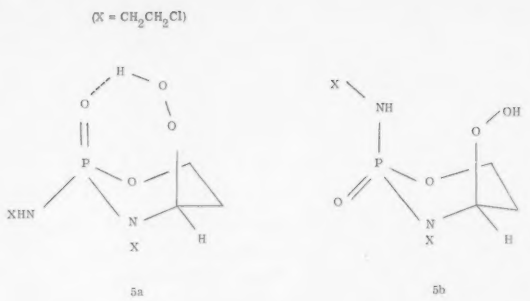


FIGURE 5

It is important to note that the P-N-C_4-H coupling constants of *N*-unsubstituted CP derivatives (*3,4*) are greater than those of IP derivatives, as shown in table 1. The greatest value observed for the dimeric peroxide (*4*) is presumably attributable to the intramolecular hydrogen bonds between the P = O and H-N groups (19), which may be responsible for an effective dπ-pπ overlapping between P and the ring nitrogen (N_3) (20). In the case of *N*-substituted 4-hydroperoxy-CPs (*14,15*), however, the P-N-C_4-H coupling constants are considerably decreased, being comparable to those of IP derivatives. These nmr differences may be attributed to a change of the P-N-C_4-H dihedral angle, or more likely to a change of hybridization at N_3. The X-ray analysis of 4-hydroperoxy-CP (*3*) and 4-hydroperoxy-IPs (*5a* and *5b*) has revealed that the N_3 of *3* deviates from trigonal configuration, while *5a* and *5b* have an exactly sp^2 hybridization at N_3.[5]

Metabolism

Some recent reports (21-24) have suggested that IP, like CP, is metabolized via two different pathways after initial C_4-hydroxylation, the first being further oxidation to 4-keto-IP (*6*) and carboxyisophosphamide (carboxy-IP) (*17*) and the second being decomposition to the cytotoxic fragments *N,N'*-bis(2-chloroethyl)phosphorodiamidic acid (*22*) and acrolein. In a recent communication, however, we reported that 4-hydroperoxy-IP (*5a*) showed

[5]Camerman A. Personal communication.

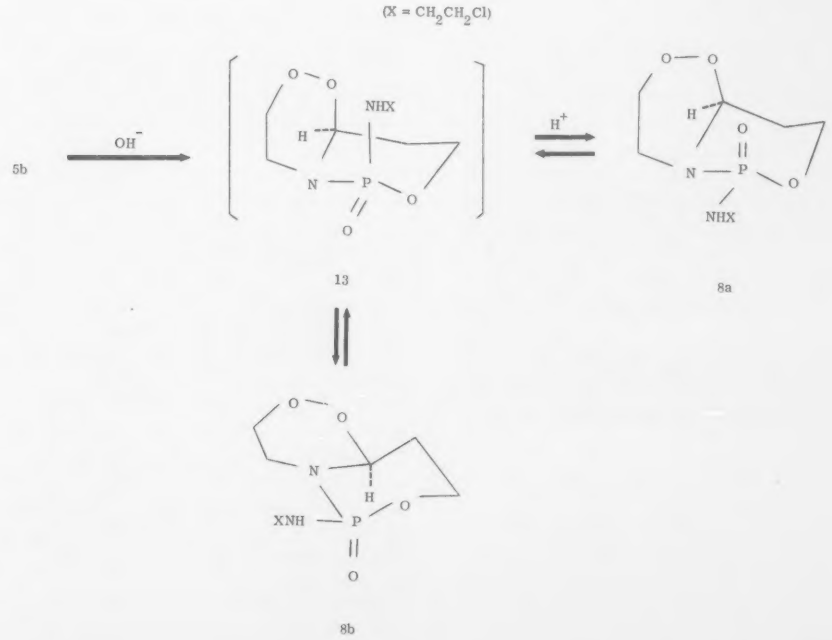


FIGURE 6

significantly different metabolic behavior from that of the parent IP (3). In the case of IP (*16*), three characterizable metabolites (*17*, *18*, and *19*) were isolated, accompanied by a considerable amount of unmetabolized *16*, from rabbit urine collected 24 hours after administration of the drug. The approximate ratios of the amounts of the metabolites isolated were *17:18:19:16* = 10:5:3:4. The structures of *N*-dealkylated metabolites *18* and *19* were confirmed by synthesis, and carboxy-IP (*17*) was characterized by conversion into a crystalline 4-phenylphenacyl ester *20* (mp 109°C-112°C). The formation of the *N*-dealkylated metabolites *18* and *19* indicates that the in vivo C_4-oxidation of IP is in competition with oxidation of the side-chain carbons, a situation which would account for the less efficient activation of the drug in vivo. On the other hand, 4-hydroperoxy-IP (*5a*) was metabolized into *17*, *6*, and a new five-membered compound [*N*-(2-chloroethyl)-3-(2-oxo-1, 3, 2-oxazaphospholidin-1-yloxy)propionamide] (*21*) in the ratio of *17:21:6* = 40:8:1. The epimeric 4-hydroperoxy-IP (*5b*) was also found to be metabolized into these compounds in essentially the same ratios as found for *5a*. In each of these experiments, no *N*-dechloroethylated metabolite was isolated, while the presence of very polar 4-(*p*-nitrobenzyl)pyridine-positive compounds was detected by thin-layer chromatography of the native urine. However, the presence of *N,N'*-bis(2-chloroethyl)phosphorodiamidic acid (*22*) in the native urine could not be confirmed, and results of bioassay experiments indicated that the amount of the cytotoxic acid *22* in the urine (if present at all) was negligible. The five-membered metabolite *21* was found to be a major metabolite of 4-keto-IP (*6*), suggesting a new metabolic pathway involving intramolecular nucleophilic displacement at phosphorus via an intermediate *23*, a proposal which has been supported chemically (3). The fact that no *N*-dealkylated metabolite is formed from both epimers *5a* and *5b*, as well as their equal metabolic behavior, is somewhat surprising. This suggests that the enzymatic *N*-dealkylation is inhibited by an oxygen functionality at C_4, and that the phosphorus stereochemistry has no significant effect upon the in vivo metabolic reaction of the C_4-oxidized IP derivatives, although at present we can tell nothing about the stereochemistry of an activated species of IP produced in vivo (fig 7).

In Vitro Cytotoxicity

The in vitro cytotoxic effect of IP derivatives was tested against HeLa and L1210 leukemia cells in free-floating culture.

The HeLa cells were grown in Eagle's MEM supplemented with 10% bovine serum. The L1210 leukemia cells were cultivated in MEM augmented with 10% fetal calf serum. The determination of

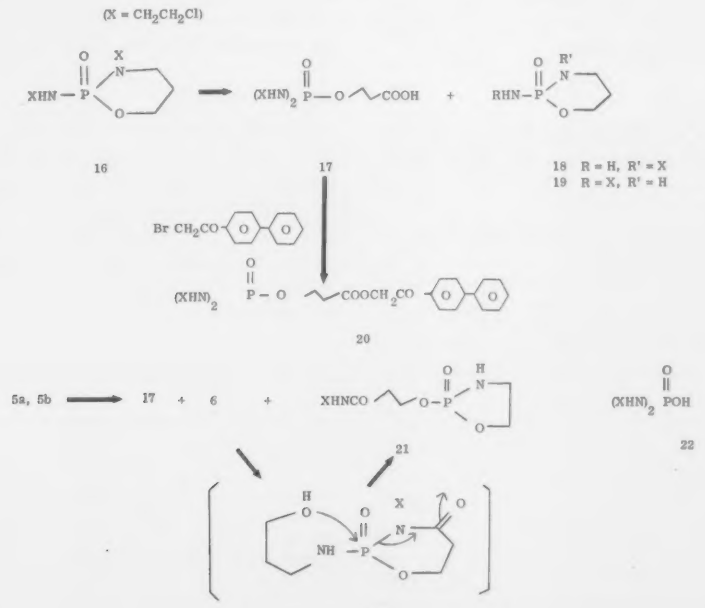


FIGURE 7

cytotoxicity was based on measurement of the inhibition of cell growth according to the previously described method (2). The results obtained are listed in table 2. As clearly shown, the epimeric 4-hydroperoxy-IP (*5b*) was most toxic on the L1210 leukemia cells (ED50 0.8 μg/ml), followed by *5a* (ED50 1.8 μg/ml). CP was least toxic (ED50 > 100 μg/ml). A similar order of susceptibility was found with HeLa cells. The HeLa cells were exposed to the drugs for 48 hours and the cell number was determined. With L1210 leukemia cells the cell number was counted 72 hours after addition of the drugs.

In Vivo Antitumor Activity

Methods

Five-week-old BDF_1 and ddN mice were used. The test groups consisted of four male and four female mice; the control groups consisted of five males and five females. The BDF_1 mice were inoculated ip with 10^5 cells of L1210 leukemia suspended in saline; the ddN mice were inoculated with 10^7 cells of Ehrlich carcinoma in saline. Five, 5-week-old Wistar rats were inoculated sc in the back with 10^7 cells of Yoshida ascites tumor. Aqueous solutions of 4-hydroperoxy-IPs *5a* and *5b* were given ip once on the day following tumor inoculation. The percent increase in lifespan over controls was calculated, and the tumor growth inhibition rate (treated/control) was calculated from the tumor weight on the 7th day after inoculation.

Results

Mean survival time (MST) in the control group after L1210 cell inoculation was 8.1 days. Mice given 5 mg/kg of *5a* survived 11.2 days. Seven of eight mice receiving 25 mg/kg survived > 30 days as did all mice receiving 50 and 100 mg/kg and five of eight mice given 200 mg/kg. Toxicity was observed in mice given > 200 mg/kg of *5a*.

The survival time of mice given *5b* was 11.5 days at 5 mg/kg. Six of eight mice given 25 mg/kg and all mice receiving 50 and 100 mg/kg survived > 30 days. Administration of 200 mg/kg caused toxic symptoms. The above results show that both the activity and toxicity of the compounds are almost the same (fig 8).

The MST of control mice inoculated with Ehrlich ascites carcinoma was 14.5 days. The survival time was increased slightly (16.4 days) in mice given 10 mg/kg of *5a* and was 21.0 days in the 25-mg/kg dosage group. Three of eight mice given 100 mg/kg survived > 30 days and toxicity was seen at 200 mg/kg. A lifespan of 19.6 days was seen in the group given 5 mg/kg of *5b*. The survival time increased with increasing doses of *5b*. Six of seven mice in the 100-mg/kg group survived for > 30 days and toxicity was seen at > 200 mg/kg.

These results clearly show that the activity of *5b* against Ehrlich ascites carcinoma is higher than that of *5a* (fig 9).

5a and *5b* showed about the same activity against the Yoshida solid tumor as follows: tumor growth was perfectly controlled by 4 mg/kg, 90%-95% by 2 mg/kg, 50% by 1 mg/kg, 15%-20% by 0.5 mg/kg, 5%-10% by 0.25 mg/kg, and 0 by 0.125 mg/kg administration of either drug (fig 10).

Acute Toxicity

Five-week-old BDF_1 male mice and Wistar female rats were used. Aqueous solutions of the compounds were administered iv and ip. In the mice, the LD50 by either route was 220 mg/kg for *5a* and 250 mg/kg for *5b*. In the rats, the LD50 was 250 mg/kg by either route for *5a* and 220 mg/kg by iv administration and 180 mg/kg by ip administration for *5b* (table 3).

TABLE 2.—Comparison of the ED50 values in HeLa and L1210 leukemia cells

Compound	ED50 (μg/ml)	
	HeLa*	L1210†
CP	>100	>100
5a	3.5	1.8
5b	3.0	0.8
7a	2.0	0.9
7b	2.7	0.4

*Cells were exposed to the drugs for 48 hrs and cell number was determined.

†Cell number was counted 72 hrs after addition of the drugs.

TABLE 3.—Acute toxicity of *5a* and *5b* in mice and rats: LD50 (mg/kg)

Animal	Route	LD50	
		5a	*5b*
Mouse, BDF_1, male	iv	220	230
	ip	220	230
Rat, Wistar, female	iv	250	220
	ip	250	180

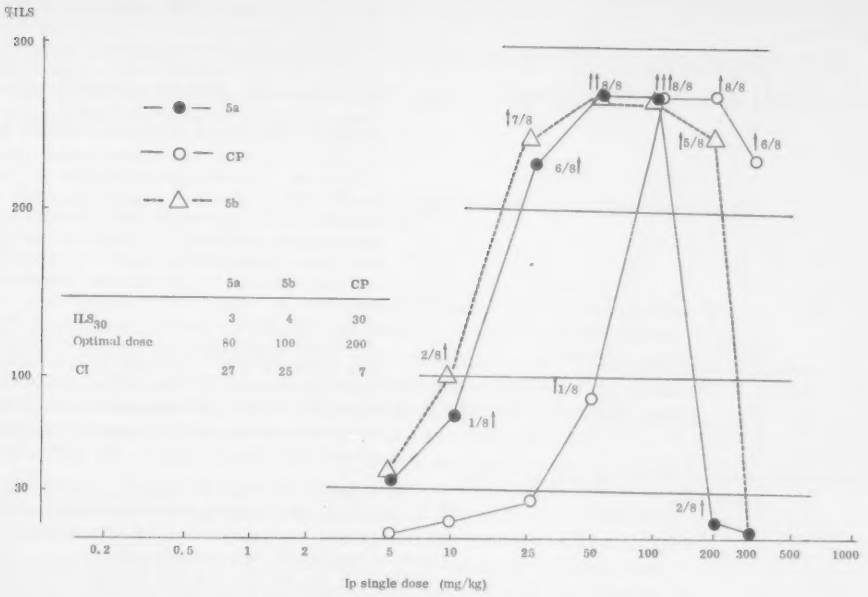


FIGURE 8.—Comparison of lifespan activity against L1210 leukemia cells.

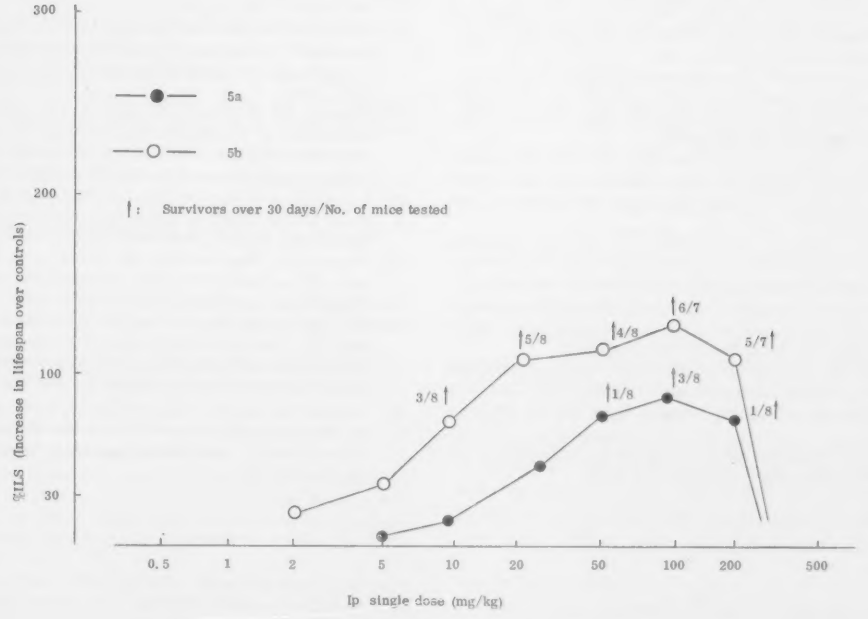


FIGURE 9.—Dose-response curves of *5a* and *5b* against Ehrlich ascites carcinoma.

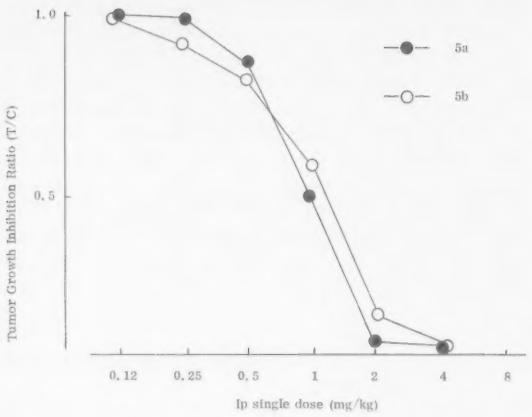


FIGURE 10.—Dose-response curves of ***5a*** and ***5b*** against Yoshida solid tumor.

REFERENCES

1. TORKELSON AR, LABUDDE JA, and WEIKEL JH, JR. The metabolic fate of cyclophosphamide. Drug Metab Rev 3:131-165, 1974.
2. TAKAMIZAWA A, MATSUMOTO S, IWATA T, ET AL. Studies on cyclophosphamide metabolites and their related compounds. 2. Preparation of an active species of cyclophosphamide and related compounds. J Med Chem 18:376-383, 1975.
3. ——. Synthesis and metabolic behavior of the suggested active species of isophosphamide having cytostatic activity. J Med Chem 17:1237-1239, 1974.
4. CAMERMAN A, SMITH HW, and CAMERMAN N. Stereochemistry of activated cyclophosphamide antitumor drugs: molecular structure of 4-hydroperoxycyclophosphamide. Biochem Biophys Res Commun 65:828-832, 1975.
5. TAKAMIZAWA A, MATSUMOTO S, and IWATA T. 4-Hydroxycyclophosphamide anhydro-dimer: revised structure of the Fenton oxidation product of cyclophosphamide. Tetrahedron Lett 6:517-520, 1974.
6. STRUCK RF, THORPE MC, COBURN WC, JR, ET AL. Cyclophosphamide. Complete inhibition of murine leukemia L1210 in vivo by a Fenton oxidation product. J Am Chem Soc 96:313-315, 1974.
7. VAN DER STEEN J, TIMMER EC, WESTRA JG, ET AL. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
8. EDMUNDSON RS, and MITCHELL EW. Cyclic organophosphorus compounds. Part XI. Stereoisomeric aralkyl phosphonates of the 1,3,2-dioxaphosphorinane series. J Chem Soc (C), 1970, pp 752-759.
9. HALL DL, and MALCOLM RB. A novel class of isomeric phosphate esters. Can J Chem 50:2102-2110, 1972.
10. BENTRUDE WG, and TAN HW. Conformations of saturated cyclic phosphorus heterocycles. II. 5-*tert*-Butyl-2-amino-1,3,2-dioxaphosphorinanes. Apparent effects of P - N vicinal interactions on the conformational energy of amino groups on trivalent phosphorus and the influence of lone pair orientation on $^3J_{HeqP}$. J Am Chem Soc 95:4666-4675, 1973.
11. MOSBO JA, and VERKADE JG. Stereochemistry of oxidation of trivalent phosphorus and configurational assignments in 2-substituted 1,3,2-dioxaphosphorinanes. J Am Chem Soc 95:4659-4665, 1973.
12. STEC W, and MIKOLAJCZYK M. Stereochemistry of organophosphorus cyclic compounds. II. Stereospecific synthesis of *cis*- and *trans*-2-halogeno-2-oxo-4-methyl-1,3,2-dioxaphosphorinans and their chemical transformations. Tetrahedron 29:539-546, 1973.
13. MIKOLAJCZYK M, KRZYWANSKI J, and ZIEMNICKA B. Diastereomeric 2-fluoro-4-methyl-1,3,2-dioxaphosphorinan-2-thiones. Retention stereochemistry in nucleophilic substitution at thiophosphoryl phosphorus. Tetrahedron Lett 19:1607-1610, 1975.
14. WHITE DW, BERTRAND RD, MCEWEN GK, ET AL. Structural implications of nuclear magnetic resonance studies on 1-*R*-1-phospha-2,6-dioxacyclohexanes. J Am Chem Soc 92:7125-7135, 1970.
15. WHITE DW, MCEWEN GK, BERTRAND RD, ET AL. Structural implications of nuclear magnetic resonance and infrared investigations on 2-substituted 1,3,2-dioxaphosphorinan-2-ones. J Chem Soc (B), 1971, pp 1454-1461.
16. HALL DL, and MALCOLM RB. Studies of organophosphorus derivatives. Part I. Nuclear magnetic resonance studies of the 2-oxo-1,3,2-dioxaphosphorinane system. Can J Chem 50:2092-2101, 1972.
17. EDMUNDSON RS. Cyclic organophosphorus compounds. Part XIV. A proton magnetic resonance study of the stereochemistry of some 5,5-di- and 4,5,5-trisubstituted 1,3,2-dioxaphosph(v)orinans. J Chem Soc (C), 1972, pp 1660-1666.
18. COOPER DB, HARRISON JM, and INCH TD. Preparation, stereochemistry and some reactions of 1,3,2-oxazaphospholanes. Tetrahedron Lett 31:2697-2700, 1974.
19. STERNGLANZ H, EINSPAHR HM, and BUGG CE. Structure and conformation of 4-peroxycyclophosphamide. A cytotoxic oxidation product of cyclophosphamide. J Am Chem Soc 96:4014-4015, 1974.
20. HUTCHINS RO, MARYANOFF BE, ALBRAND JP, ET AL. Conformational analysis of phosphorus heterocycles. ^1H and ^{31}P nuclear magnetic resonance study of *N,N'*-dimethyl-(2*R*)-2-phospho-1,3-diazacyclohexanes. J Am Chem Soc 94:9151-9158, 1972.
21. ALLEN LM, and CREAVEN PJ. In vitro activation of isophosphamide (NSC-109724), a new oxazaphosphorine, by rat liver microsomes. Cancer Chemother Rep 56:603-610, 1972.
22. ALARCON RA, MEIENHOFER J, and ATHERTON E. Isophosphamide as a new acrolein-producing antineoplastic isomer of cyclophosphamide. Cancer Res 32:2519-2523, 1972.
23. HILL DL, LASTER WR, JR, KIRK MC, ET AL. Metabolism of iphosphamide (2-(2-chloroethylamino)-3-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide) and production of a toxic iphosphamide metabolite. Cancer Res 33:1016-1022, 1973.
24. CONNORS TA, COX PJ, FARMER PB, ET AL. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.

Two Stable Fenton Oxidation Products of Cyclophosphamide (NSC-26271) as Precursors of 4-Hydroxycyclophosphamide (NSC-196562) Under Physiologic Conditions[1]

Catherine Benckhuysen,* Jan van der Steen, and Edmond J. Spanjersberg[2,3]

SUMMARY

Cytostatic and chemical properties of two cyclophosphamide (CP) 4-peroxides, 4-hydroperoxycyclophosphamide and 4-hydroxycyclophosphamide anhydro-dimer, that were earlier isolated and identified after Fenton oxidation of CP, are summarized and discussed. Their cytostatic toxicities, which are quantitatively comparable to those of the primary metabolite of CP, 4-hydroxycyclophosphamide, are explained from their apparent spontaneous conversion to mono-oxidized forms of CP, both in vitro and in vivo. The conversion is shown to proceed via autocatalyzed reactions, which are thought to imply the occurrence of free radicals during deoxygenation. The point is raised whether any qualitative difference in cytotoxicity might be caused by the action of these free radicals. Current dose-effect studies of residual colony-forming ability after treatment of 3T6 mouse fibroblasts and Yoshida sarcoma cells in suspension culture are introduced. Further studies on the local toxic action of the radicals under in vivo conditions are necessary if the peroxides are to be introduced in local or regional clinical treatments.

[Cancer Treat Rep 60:369-372, 1976]

Cyclophosphamide (CP) (I, fig 1) is an interesting substrate for oxidation in the Fenton model system for the drug-metabolizing enzymes. The system performs hydroxylation of aromatic and heteroaromatic compounds, presumably through the action of hydroxyl radicals, although it has been assumed that perhydroxyl radicals also participate (1).

When we applied the Fenton oxidation system to CP the first product to be isolated and identified was 4-ketocyclophosphamide (4-keto-CP) (III, fig 1) (2), one of the urinary metabolites. While this suggests a relationship between the Fenton oxidation of CP and its in vivo metabolism, no definite proof could be obtained (with the techniques that we employed) that the metabolically known hydroxylation at carbon 4 also occurs in Fenton's system, although there are certainly indications for that. On the other hand, the oxidation of CP in the Fenton system led us to two compounds that did not appear to be directly related to the metabolism of the drug. Common to these oxidation products was that they were more abundantly formed in the reaction mixtures if a 1.5 M ratio of hydrogen peroxide to ferrous ions was applied for oxidation, instead of the usual equal molarity.[4] When extracted into dichloromethane one of the two products was found to be converted to the other on reflux. The compounds were isolated and both proved to have a carbon-4 peroxide structure; the one acting as the precursor was identified as 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP) (V, fig 2) (3), and the other was identified as 4-peroxycyclo-

[1]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

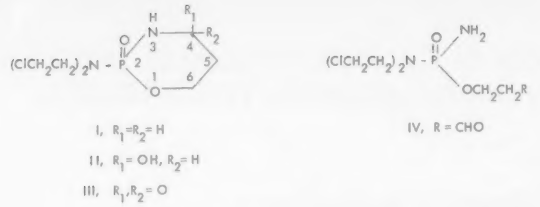
4-Hydroxycyclophosphamide: CAS reg. No. 40277-05-2; 2*H*-1,3,2-oxazaphosphorin-4-ol, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide.

[2]Department of Biochemistry, Antoni van Leeuwenhoek huis, The Netherlands Cancer Institute, Amsterdam.

[3]The competent experimentation by Miss S. C. van 't Hoff in the in vivo toxicity studies is gratefully acknowledged. We also wish to thank Dr. L. A. Smets, Department of Experimental Cytology, Antoni van Leeuwenhoekhuis, The Netherlands Cancer Institute, for his interest.

**Reprint requests to:* Dr. Catherine Benckhuysen, Department of Biochemistry, Antoni van Leeuwenhoekhuis, The Netherlands Cancer Institute, Sarphatistraat 108, Amsterdam, The Netherlands.

[4]The reaction mixtures contained CP (40 mM), H_2O_2 (117 mM), $FeSO_4$ (81 mM), unbuffered solution of 20°C; oxidation was taken to occur instantaneously.



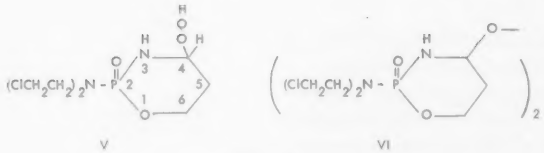
I, $R_1=R_2=H$

II, $R_1=OH$, $R_2=H$

III, $R_1,R_2=O$

IV, R = CHO

FIGURE 1



V

VI

FIGURE 2

phosphamide or 4-hydroxycyclophosphamide anhydro-dimer (VI, fig 2). The latter structure was ultimately recognized by Takamizawa et al (4), who added to our data and their own corresponding spectral data those of molecular weight determination.

In the Fenton system, it is to be expected that augmenting the relative amount of hydrogen peroxide will promote the formation of perhydroxyl radicals (5). Although there are few reports in the literature on peroxides that occur as intermediates during microsomal hydroxylation (6,7), there is at the moment little reason to suppose that CP 4-peroxides do occur in vivo. Certainly, we did not find free 4-hydroperoxy-CP in our studies of the metabolism of CP in the isolated perfused rat liver.

Apart from this point, the peroxides are interesting substances because they exhibit a high cytostatic activity both in vitro and in vivo as has by now been reported by several authors (3,8-10). When quantitatively compared, the cytostatic effectiveness of both peroxides against BHK cells in vitro was very similar to that of 4-hydroxy-CP prepared by reduction of 4-hydroperoxy-CP according to Takamizawa et al (8). In each of two experiments a pair of compounds was comparatively studied. Steep dose-effect curves were found and essentially no differences were noticed in the ED50 values of the three agents. Drug-treated media were not replaced during the 48-hour growth period.

In their chemical behavior in aqueous solution the peroxides show a close resemblance to 4-hydroxy-CP. The first point to be noticed was that the acrolein hydrazone was formed when either a fresh or an aged solution of a peroxide was treated with 2,4-dinitrophenyl hydrazine. Also, it could be shown by gas chromatography that the theoretical amount of acrolein was released in 4-5 hours from either peroxide when a 1 mM aqueous solution was kept at 37°C and pH 7.2. The release of acrolein under these conditions implies a spontaneous breakdown via aldophosphamide (IV, fig 1).

The occurrence of 4-hydroxy-CP in a similar solution of the hydroperoxide is suggested by thin-layer chromatography (TLC) on silica gel of its dichloromethane extract (solvent system CH_2Cl_2:*n*-BuOH; 9:1) but cannot be stated to be unequivocally demonstrated with this technique. Good evidence for the occurrence of 4-hydroxy-CP has been presented by Voelcker et al (11). An aqueous solution of the dimer also seems to contain 4-hydroxy-CP, as evidenced by TLC. Soon after dissolving the dimer, 4-hydroperoxy-CP was also encountered.

Because of the conversion of the peroxides to other alkylating species, we determined, by reaction with 4-(4′-nitrobenzyl)pyridine (NBP), the course of the alkylating activity of a peroxide solution with time. In the case of 4-hydroxy-CP this activity remains constant for some 45 minutes after which it gradually declines, but for the peroxides an initial increase of some 50% was found. The increase set in immediately for the dimer but only after some 30 minutes in the case of the hydroperoxide. These initial differences in alkylating activity of the solutions did not however have much bearing on the overall cytostatic effect of the compounds. Incubation of BHK cells with drugs for 30 minutes did not change the cytostatic activity of the dimer relative to that of 4-hydroxy-CP and hardly affected that of the hydroperoxide.

The peroxides can both be shown by TLC to persist for a considerable time under pseudophysiologic conditions in vitro. Persistence in vivo was monitored in mice injected ip with a dose of 1 mmol/kg following the ip administration of a 6% solution of dextran (0.1 ml/kg body weight). Samples of this artificial ascitic fluid were then analyzed with a highly standardized TLC procedure. After 2 hours the peroxides were both recovered to the extent of 5% of the administered dose, which agrees very well with the data obtained for a 1 mM solution in vitro. The similarity of the in vivo and in vitro data suggests that the peroxides are not subjected to enzymatic reduction to mono-oxidized forms of CP.

The conversion of the peroxides to 4-hydroxy-CP presumably occurs via radical reactions. This is suggested by the results in figure 3. The time-course of the release of acrolein from the hydroperoxide and the dimer in aqueous solution at pH 7.2 and 37°C was determined. In both cases a lag-phase was found after which the reaction proceeded at a constant speed. Recently it has been shown

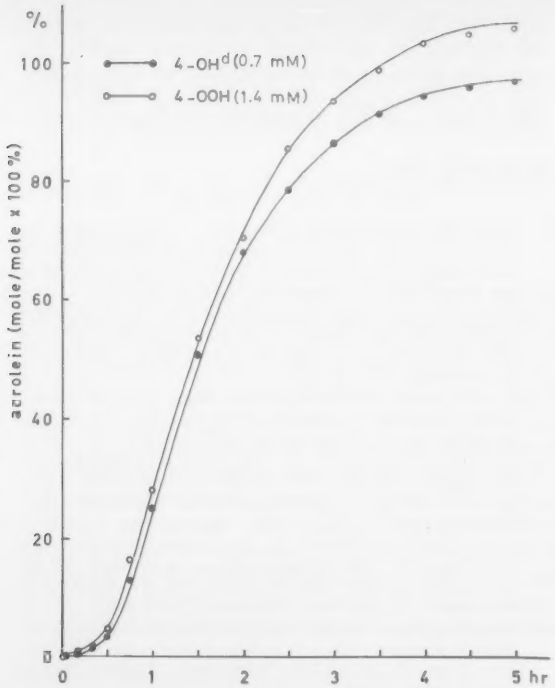


FIGURE 3.—Time-course of the release of acrolein from 4-hydroperoxy-CP (4-OOH) and 4-hydroxy-CP anhydro-dimer (4-OH^d) in phosphate buffer (pH 7.2 at 37°C).

by Dr. Hohorst's group that under the same conditions as ours, the release of acrolein from 4-hydroxy-CP, in contrast to that from the hydroperoxide, proceeds at a constant speed from zero time (11). From these kinetic data it is assumed that the deoxygenation of the peroxides occurs via autocatalyzed radical reactions.

In figure 4 the reactions that are likely to occur are summarized. A comparison between the two peroxides indicates that breakdown of the dimer will give rise to relatively half as many free hydroxyl radicals as does the hydroperoxide.

Because of the reactivity of free radicals, the consequences of their occurrence both in vivo and in vitro during spontaneous deoxygenation of the peroxides should be considered if one is to use the peroxy compounds as more stable substitutes for the rather labile 4-hydroxy-CP. If the overall cytotoxicity of the peroxides was qualitatively different from that of 4-hydroxy-CP this might be discernible from the shape of the dose-survival curves in which residual colony-forming ability of treated cells is plotted in log units against dose. Such dose-survival curves are not only characterized by the slopes of their straight portions, but also by

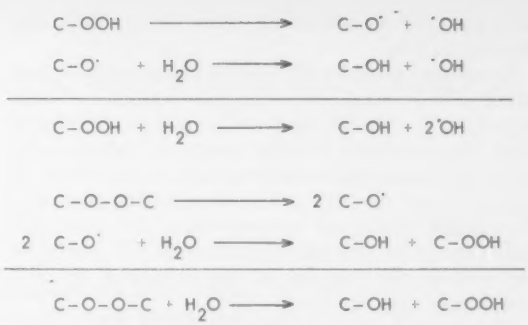


FIGURE 4.—Generation of OH radicals during conversion of CP 4-peroxides.

the occurrence of an initial shoulder of varying broadness. While the first criterion quantifies cytotoxic effectiveness the occurrence of a shoulder may reveal something about the ability of a system to counteract or prevent the lethal action of low concentrations of the drug. Thus, differently shaped shoulders indicate qualitative differences in the interaction between cell and drug. To compare the characteristic features of the cytotoxic actions of both peroxides and 4-hydroxy-CP, we recently started such cell-survival studies in vitro with two different cell culture systems.

3T6 mouse fibroblasts with a high plating efficiency when grown in the presence of lethally irradiated feeder cells were treated for 1 hour in tissue culture medium with either 4-hydroperoxy-CP (two experiments) or 4-hydroxy-CP (one experiment). After 6-7 days colonies were counted after staining with hematoxylin. The cells were not very sensitive. A 50% decrease in survival was reached at 4.2 μg/ml of 4-hydroxy-CP; to lower the surviving fraction by 4 decades 15 μg/ml of this agent was needed. It is too early to make a comparison between the data for the two agents; if reproducibility is indeed that high, there must be some difference both in the broadness of the shoulder and the slope of the curves, the curve of 4-hydroperoxy-CP being the somewhat steeper one.

Suspension cultures of Yoshida sarcoma cells were developed according to Fox and Fox (12). The fraction of cells surviving after treatment was determined by the back-extrapolation method of Alexander and Mikulski (13). The system proved highly sensitive to the hydroperoxide, which at 1 μg/ml decreased the surviving fraction by more than 4 decades. A shoulder in the curve is lacking and it will be interesting to see if 4-hydroxy-CP will present a similarly shaped line. If anything was suggested by the companion pilot experiment with 4-hydroxy-CP, it would be that in this system

4-hydroxy-CP is somewhat more active than the hydroperoxide.

In clinical practice a cytostatically active equivalent of CP would be especially useful for local injection into the pleural cavity, or for administration via the intra-arterial route in infusions or even perfusions of the extremities. In all those instances local toxicity is the threatening complication and it should not be overlooked that free radicals formed by the deoxygenation of CP peroxides will add to the toxicity of the peroxides towards the normal tissues.

The limited data on the toxicity of 4-hydroperoxy-CP available up to now have mainly been obtained after iv administration. We compared the toxicities of 4-hydroperoxy-CP and CP given ip on 5 consecutive days to male hybrids of two inbred rat lines that are maintained at our Institute. A high LD10 was found for CP (215 μmols/kg/day) compared to 93 μmols/kg for 4-hydroperoxy-CP. In the case of the hydroperoxide a late cause of death was an accumulation of a hemorrhagic ascitic fluid and multiple adhesions in the peritoneal cavity. Such deaths were observed starting on Day 26 and occurred at doses that were only slightly above the LD10. Microscopically, a granulomatous thickening of the peritoneum was noticed.

It is not suggested that the local concentration of free radicals was high enough to cause those ip reactions. First, it is necessary to estimate to what extent the lesions may have been caused by the alkylating groups, the radical reactivity, or both. The only point to be stressed at the moment is that studies on acute toxicity should be extended over quite long periods and should also cover signs of local toxicity if the agents are to be used locally or regionally in clinical treatments.

Considering the possible consequences of radical formation during deoxygenation we were led to an attempt to reduce the hydroperoxide to 4-hydroxy-CP in its vehicle prior to administration in vivo or in vitro. Thirty minutes after adding ascorbic acid to a solution of the hydroperoxide in phosphate-buffered saline (molar ratio, 5:1), the 4-hydroperoxy-CP had nearly completely disappeared from the solution as was demonstrated by TLC. On the other hand, the cytotoxic effect of the hydroperoxide, determined at 50 μM (15 μg/ml), against BHK cells was not diminished to any degree, while ascorbic acid alone had no effect on cell growth. A procedure like this would be convenient for practical application, and seems to deserve further exploration in view of the reliability of CP 4-peroxides as substituents for 4-hydroxy-CP both in experimental work and in clinical practice.

REFERENCES

1. Staudinger HJ, Kerekjártó B, Ullrich V, et al. A study on the mechanism of microsomal hydroxylation. *In* Oxidases and Related Redox Systems (King TE, Mason HS, and Morrison M, eds). New York, John Wiley, 1965, vol 2, pp 815-837.
2. Benckhuysen C, van der Steen J, and Westra JG. Activation of cyclophosphamide (Endoxan) in the Fenton reaction. (Abstr B-3/9) *In* Proceedings of the 8th International Congress of Chemotherapy, Prague, 1971.
3. van der Steen J, Timmer EC, Westra JG, et al. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
4. Takamizawa A, Matsumoto S, and Iwata T. 4-Hydroxycyclophosphamide anhydro-dimer: revised structure of the Fenton oxidation product of cyclophosphamide. Tetrahedron Lett 6:517-520, 1974.
5. Norman ROC, and Smith L, Jr. Mechanisms of aromatic hydroxylation and ring-opening reactions. *In* Oxidases and Related Redox Systems (King TE, Mason HS, and Morrison M, eds). New York, John Wiley, 1965, vol 1, pp 131-156.
6. Chen C, and Lin CC. Mechanism of aliphatic hydroxylation. Tetralin hydroperoxide as an intermediate in the hydroxylation of tetralin in rat-liver homogenate. Biochim Biophys Acta 170:366-374, 1968.
7. Chen C, and Lin CC. [9-^{14}C]fluorene hydroperoxide as a possible intermediate in the hydroxylation of [9-^{14}C]fluorene by rat liver homogenate. Biochim Biophys Acta 184:634-640, 1969.
8. Takamizawa A, Matsumoto S, Iwata T, et al. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
9. Takamizawa A, Matsumoto S, Iwata T, et al. Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and related compounds. J Med Chem 18:376-383, 1975.
10. Struck RF, Thorpe MC, Coburn WC, Jr, et al. Cyclophosphamide. Complete inhibition of murine leukemia L1210 in vivo by a Fenton oxidation product. J Am Chem Soc 96:313-315, 1974.
11. Voelcker G, Draeger U, Peter G, et al. Studien zum Spontanzerfall von 4-Hydroxycyclophosphamid und 4-Hydroperoxycyclophosphamid mit Hilfe der Dünnschichtchromatographie. Arzneim Forsch 24:1172-1176, 1974.
12. Fox M, and Fox BW. The establishment of cloned cell lines from Yoshida sarcomas having differential sensitivities to methylene dimethane sulphonate in vivo and their cross-sensitivity to X-rays, UV and other alkylating agents. Chem Biol Interact 4:363-375, 1972.
13. Alexander P, and Mikulski ZB. Differences in the response of leukaemia cells in tissue culture to nitrogen mustard and to dimethyl Myleran. Biochem Pharmacol 5:275-282, 1961.

Biologic Activity of Two Derivatives and Six Possible Metabolites of Cyclophosphamide (NSC-26271)[1]

P. Lelieveld* and L. M. van Putten[2,3]

SUMMARY

The biologic activity in terms of survival of normal hematopoietic stem, osteosarcoma, and L1210 leukemia cells was determined for the following compounds: cyclophosphamide, its derivatives isophosphamide and trophosphamide, its possible metabolites *nor*-nitrogen mustard, hydroxylamine mustard, 4-ketocyclophosphamide, and acrolein, and two substitutes for its primary active metabolite 4-hydroxycyclophosphamide anhydro-dimer (4-hydroxy-CP-anhydro-dimer) and 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP). On a molar basis none of the compounds shows a better therapeutic ratio between osteosarcoma and bone marrow stem cells than the parent compound. The therapeutic ratio between L1210 leukemia and normal cells is slightly better for 4-hydroperoxy-CP only. It may be concluded that the conversion of 4-hydroxy-CP-anhydro-dimer and 4-hydroperoxy-CP to the primary active metabolite 4-hydroxycyclophosphamide differs quantitatively. Moreover, it appears that by the use of these precursors a better therapeutic ratio might be obtained for some malignancies but not for others.

[Cancer Treat Rep 60:373-379, 1976]

The aim of model studies with chemotherapeutic agents is to find better ways to cure patients of their malignant tumors. To attain this goal all malignant cells must be killed or sterilized. At the same time it is of importance that the number of normal cells is not reduced below a critical value. Thus, it is necessary to find agents that are more effective in killing malignant cells than normal cells.

An example of such an agent is cyclophosphamide (CP). After its administration to animals or man a number of metabolites with alkylating properties have been shown in the serum and urine. The various steps in activation of CP are described extensively by other authors in this symposium. Up to the present the most effective antitumor metabolite has not been identified with certainty. Since it is likely that this metabolite will show at least the same effectiveness on tumor cells as CP itself, its identification seems desirable.

In this report studies on the effects of CP, two of its derivatives, four possible metabolites, and two substitutes for its primary active metabolite on the survival of normal and malignant cells are described in an attempt to identify a metabolite with a higher relative biologic effectiveness than the parent compound.

MATERIALS AND METHODS

The relative toxicity on tumor and normal cells served as a criterion for the identification of the most effective metabolite, which on a molar basis is assumed to show at least the same relative effectiveness as CP.

Chemotherapeutic Agents

Besides CP itself, which was studied earlier (1), we investigated the following compounds: isophosphamide (IP), trophosphamide, *nor*-nitrogen mustard, hydroxylamine mustard, 4-ketocyclophosphamide (4-keto-CP), acrolein, 4-hydroxycyclophosphamide anhydro-dimer (4-hydroxy-CP-anhydro-dimer), and 4-hydroperoxycyclophosphamide

[1]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[2]Radiobiological Institute of the Organization for Health Research TNO, Rijswijk, The Netherlands.

[3]We would like to thank Metha Pijpers-de Bruin and Wil Akkerman for technical assistance, Leonie van der Burg-Verwey and Joke Snel-Ebberink for irradiating the mice, and Hetty Jense and Eric van der Reyden for preparing the manuscript.

*Reprint requests to: Dr. P. Lelieveld, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk, The Netherlands.

(4-hydroperoxy-CP).

CP was injected ip. Because insufficient data were available concerning their stability, it was decided to inject 4-hydroxy-CP-anhydro-dimer and 4-hydroperoxy-CP iv. The other agents were administered sc. Further data on the drugs used in this study are presented in table 1.

The relative toxicity after exposure in vivo to these agents was measured by the determination of the cell survival in two tumor lines and in one normal cell type. Such a comparison is essential for the therapeutic evaluation.

Assay of Survival of Hematopoietic Stem Cells After Drug Treatment

For the determination of the activity against normal cells we used hematopoietic stem cells from the mouse since bone marrow is often the tissue that defines the tolerance to many chemotherapeutic agents. The survival of hematopoietic stem cells was determined by the spleen-colony technique of Till and McCulloch (2).

Groups of four or five mice were treated at each drug dose and 16 hours later a bone-marrow suspension was prepared from their femurs. From the cell suspensions a suitable dilution was injected iv into groups of ten lethally irradiated isogenic mice. Nine days later the spleens of these recipients were fixed in Tellyesniczky's solution and the number of spleen colonies was counted. Then the number of colony-forming units (CFU) per donor femur and the surviving fraction in comparison with the untreated control were calculated.

Assay of Antitumor Effectiveness

For the determination of the activity against tumor cells we used L1210 leukemia cells, available in our laboratory since 1969, and transplantable osteosarcoma cells, the properties of which have been described earlier (3,4). To prepare a single-cell suspension of the osteosarcoma the cell dispersion technique was used as described by Reinhold (5). Tumor-cell survival was calculated with the aid of Hewitt's endpoint dilution technique (6) which was slightly modified (7). Briefly, groups of three to five mice, carrying a tumor in each flank, were treated at each drug dose. After 16 hours the tumors from each group were pooled and a single-cell suspension was prepared by mild trypsinization and agitation. The cell suspensions were counted,

TABLE 1.—Source and preparation of drugs used*

Drug	Supplier†	Vehicle
CP	Asta-Werke Aktiengesellschaft Chemische Fabrik, Brackwede, Germany	Saline
IP	Asta-Werke	Saline
Trophosphamide	Asta-Werke	Dissolved in Solketal, Orpe, and saline‡
Nor-nitrogen mustard	K and K Labs, Plainview, NY	Saline
Hydroxylamine mustard	Dr. C. Benckhuysen, Nederlands Kankerinstituut, Amsterdam	Saline
4-Keto-CP	Dr. C. Benckhuysen	Suspended in 2% carboxymethylcellulose
Acrolein	Merck, Germany	Saline
4-Hydroxy-CP-anhydro-dimer	Dr. C. Benckhuysen	Dissolved in dimethylsulfoxide, Orpe, and saline
4-Hydroperoxy-CP	Asta-Werke through Prof. N. Brock	Dissolved in dimethylsulfoxide, ethanol, and saline

*All solutions and suspensions were prepared within 30 mins before injection. Drugs were stored at 4°C and protected from light; 4-hydroperoxy-CP was stored at −20°C.

†Gifts of samples of these substances are gratefully acknowledged.

‡Supplier's solvents. Solketal = 2,2-dimethyl-1,3-dioxolane-4-methanol; Orpe = a polyoxy-ethylate of castor oil.

suitable dilutions were made, and serially increasing numbers of cells were injected sc into isogenic recipients. The occurrence of tumors at the inoculation sites was noted. After at least 90 days the TD50 was determined (ie, the number of cells that must be inoculated in order to produce a tumor at 50% of the injection sites). From comparison of the TD50 of treated and control tumors the surviving fraction was calculated.

Drug effectiveness against the L1210 leukemia cells was determined as follows. Mice were injected iv with 10^5 L1210 leukemia cells from an in vitro culture. After 6 days, groups of five of these leukemic mice were exposed to different types of drug treatment. Sixteen hours later the mice were killed and a bone-marrow suspension was prepared from their femurs. From this suspension, a suitable dilution was made and plated in 35-mm petri dishes containing Dulbecco's tissue culture medium supplemented with 20% (vol/vol) serum and made semisolid by the addition of 0.3% (vol/vol) agar. In addition, the L1210 leukemia cells were found to require for their growth 2-mercaptoethanol as a stimulating agent at a concentration of 60 μmol/liter. The dishes were incubated at 37°C in an atmosphere of 10% carbon dioxide and 100% humidity. After 7 days, the colonies consisting of 50 or more cells were counted and the number of leukemic CFU (culture) per femur and the surviving fraction relative to the untreated control were calculated.

Irradiation

The recipients for the spleen-colony assays were irradiated with either 750 rads of X rays from a General Electric Maxitron 250 kVp X-ray machine at an exposure rate of 55 rads/minute or with 950 rads of γ rays from a 611 curie ^{137}Cs-source at an exposure rate of 122 rads/minute.

Animals

Spleen-colony assays were carried out with (C57BL/Rij × CBA/Rij)F_1 or (C57BL/Ka × CBA/Rij)F_1 hybrids of both sexes. The survival of osteosarcoma cells was determined in (C57BL/Rij × CBA/Rij)F_1 males and the survival of L1210 leukemia cells was determined in (C57BL/Rij × DBA_2/Rij)F_1 hybrids of both sexes. In each experiment mice of only one sex were used.

RESULTS

The survival of normal hematopoietic stem and tumor cells after exposure to CP and its derivatives and metabolites was determined and dose-response curves were made. The best fitting straight line was calculated by the least squares method. Curves for the agents tested are presented in figures 1 and 2. The results are expressed on a molar basis: the surviving fraction is plotted against the dose in μmol/kg body weight. For each cell type the slopes of the survival curves were expressed as their D_0 value, ie, the dose necessary to reduce survival by 37% on the linear part of the curve. To allow equimolar comparison the D_0 value for 4-hydroxy-CP-anhydro-dimer was multiplied by two. The results are presented in table 2. A number of observations should be made from the data presented. First, acrolein is not effective in killing either bone-marrow stem or osteosarcoma cells. In this case the sensitivity of the osteosarcoma cells was determined by the capacity of the drug to induce a reduction in the volume of the tumor after sc treatment. Although the tumor volume reduction ratio obtained is a relatively poor parameter of cell survival, we have shown that it allows us to clearly differentiate between effective and ineffective agents (8). The same system was used to assay the effects of hydroxylamine mustard; this drug was also found to be inactive. Secondly, in agreement with the results seen for 4-keto-CP on stem and osteosarcoma cells, others have brought forward evidence that this agent is biologically ineffective (9,10).

It has already been mentioned that the therapeutic effectiveness of a drug depends on the relative killing of normal and malignant cells. For this reason the D_0 values from table 2 were expressed as a ratio. These relative efficiencies are presented in table 3. For CP the ratio of D_0 values for osteosarcoma to stem cells is 0.19: the osteosarcoma cells are about five times as sensitive to CP as the stem cells. The ratios for the other agents are higher, indicating a lower relative efficiency. *Nor*-nitrogen mustard is the only agent that shows greater activity towards stem than to osteosarcoma cells. The latter were not sensitive as was also shown by the tumor-volume measurements after drug treatment. The ratios of D_0 values for L1210 leukemia to stem cells did not vary markedly except for *nor*-nitrogen mustard, which is almost equally effective in stem and L1210 leukemia cells. IP and trophosphamide are less effective, but 4-hydroperoxy-CP is slightly more effective than CP. The ratios between the osteosarcoma and L1210 leukemia cells show that the two malignancies have different sensitivities to the agents, although both are rapidly proliferating and have a growth fraction of approximately 100%.

DISCUSSION

In search for the active metabolite of CP several compounds have been shown to be present after in

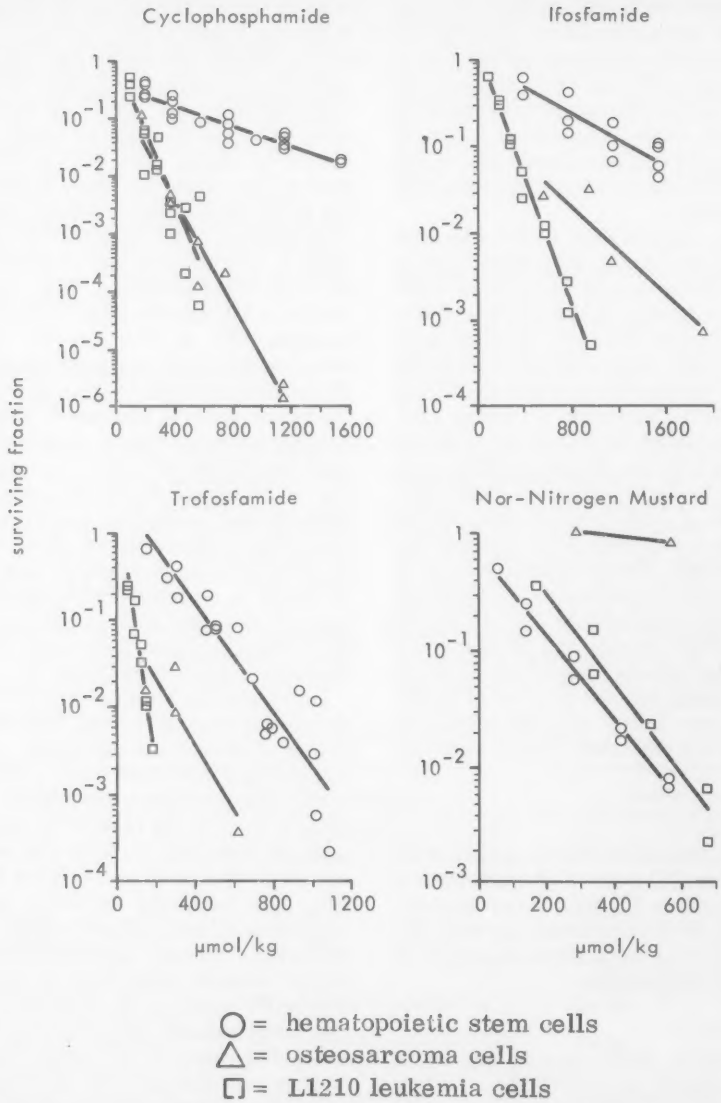


FIGURE 1.—Dose-survival curves for normal hematopoietic stem, osteosarcoma, and L1210 leukemia cells after different doses of CP, IP, trophosphamide, and *nor*-nitrogen mustard.

vivo administration or after conversion by drug-metabolizing enzymes in vitro. These findings were sufficient evidence that a mixture of metabolites is formed. This might be the consequence of the existence of different metabolic pathways. It is accepted that the first step in the activation of CP is hydroxylation at C4, yielding 4-hydroxy-CP. However, this drug was not available to us, partly because of its instability, but we were able to get two substitutes for it, 4-hydroxy-CP-anhydro-dimer and 4-hydroperoxy-CP. The former is less active in the osteosarcoma and more effective in the L1210 leukemia cells when compared to CP; the latter is equally effective in the osteosarcoma and extremely active in the L1210 leukemia cells. Both agents, however, are more toxic to bone-marrow stem cells than CP.

If we assume that the effectiveness of CP, 4-hydroxy-CP-anhydro-dimer, and 4-hydroperoxy-CP is proportional to the production of 4-hydroxy-CP, we

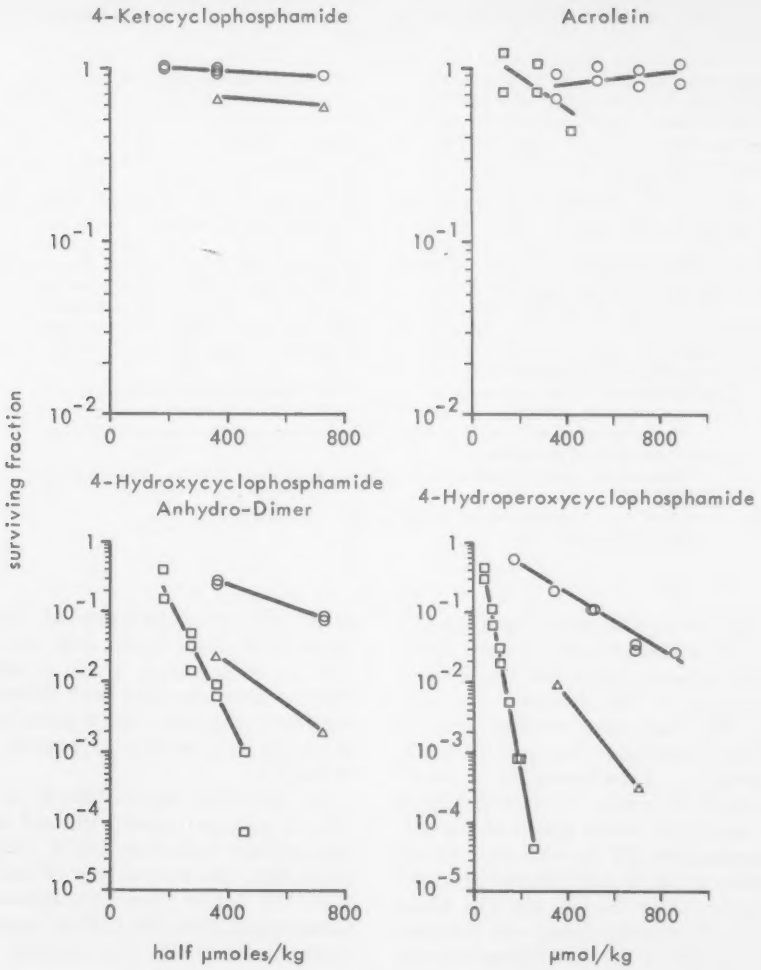


FIGURE 2.—Dose-survival curves for normal hematopoietic stem, osteosarcoma, and L1210 leukemia cells after different doses of 4-keto-CP, acrolein, 4-hydroxy-CP-anhydro-dimer, and 4-hydroperoxy-CP.

must find an explanation for the difference in hematologic toxicity of the three compounds. Such an explanation could be:

A. Incomplete transformation of CP to 4-hydroxy-CP and release in the blood stream of the latter. This is well documented for several species on the basis of excretion in the urine (11) and alkylation in the liver (12).

B. Formation of other toxic compounds from 4-hydroxy-CP-anhydro-dimer and 4-hydroperoxy-CP, which is not supported by any evidence.

C. Different rates of formation and release of 4-hydroxy-CP.

Explanation B is unlikely and A is probably correct. This has consequences for our interpretation of the results seen in the tumors. If explanation A

TABLE 2.—Effect of CP and its derivatives and metabolites on normal and malignant cells

Drug	D_0 (μmol/kg) for killing* (cell type)		
	Bone marrow (CFU)	Osteosarcoma	L1210 leukemia
CP	492	94.7	72.3
IP	579	341	120
Trophosphamide	139	114	28.2
Nor-nitrogen mustard	122	1399	112
Hydroxylamine mustard	892	Inactive†	Not done
4-Keto-CP	6116	3604	Not done
Acrolein	Inactive‡	Inactive†	240
4-Hydroxy-CP-anhydro-dimer§	298	143	51.3
4-Hydroperoxy-CP	210	100	23.0

*D_0 = dose which will lead to a reduction of cell survival by a factor of 37% on the linear part of the curve.

†Reduction in tumor volume after drug treatment showed that the compound was ineffective.

‡From the experimental data a negative slope was obtained.

§D_0 for the dimer × 2.

is correct it is easy to explain why 4-hydroxy-CP-anhydro-dimer and 4-hydroperoxy-CP are more effective in L1210 leukemia cells, but the lack of increased effectiveness in the osteosarcoma cells does not follow. The fact that neither drug is clearly better in the solid tumor may find its explanation in a changed rate of the formation of one or more intermediates (explanation C), specifically in the solid tumor leading to lower levels of the primary active intermediate. CP is activated in the liver; the activation products are released relatively slowly and reach their target via the blood stream. 4-Hydroxy-CP-anhydro-dimer and 4-hydroperoxy-CP were injected iv so that higher plasma levels of 4-hydroxy-CP or aldophosphamide may occur. The poor response of the solid tumor to these brief high levels may be explained by its poor vascular supply which is well documented (3). Further investigations with different rates of drug administration may throw some light on this point, since this may modify the ratio in the third column of table 3.

For practical application it is noteworthy that only 4-hydroperoxy-CP showed a slightly better therapeutic ratio for L1210 leukemia over stem cells. Also, the derivatives IP and trophosphamide were not better than CP. However, it should be remembered that the limited number of tumor cell types tested limits the validity of these conclusions.

TABLE 3.—Ratios of D_0 values of CP and its derivatives and metabolites for normal and malignant cells

Drug	D_0 osteosarcoma cells/D_0 stem cells	D_0 L1210 leukemia cells/D_0 stem cells	D_0 osteosarcoma cells/D_0 L1210 leukemia cells
CP	0.19	0.15	1.31
IP	0.59	0.21	2.84
Trophosphamide	0.82	0.20	4.04
Nor-nitrogen mustard	11.47	0.92	12.49
4-Hydroxy-CP-anhydro-dimer	0.48	0.17	2.79
4-Hydroperoxy-CP	0.48	0.11	4.35

REFERENCES

1. VAN PUTTEN LM, and LELIEVELD P. Factors determining cell killing by chemotherapeutic agents in vivo. I. Cyclophosphamide. Eur J Cancer 6:313-321, 1970.
2. TILL JE, and MCCULLOCH EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213-222, 1961.
3. VAN PUTTEN LM. Tumour reoxygenation during fractionated radiotherapy; studies with a transplantable mouse osteosarcoma. Eur J Cancer 4:173-182, 1968.
4. VAN PUTTEN LM, LELIEVELD P, and BROERSE JJ. Response of a poorly reoxygenating mouse osteosarcoma to X-rays and fast neutrons. Eur J Cancer 7:153-160, 1971.
5. REINHOLD HS. A cell dispersion technique for use in quantitative transplantation studies with solid tumours. Eur J Cancer 1:67-71, 1965.
6. HEWITT HB. Studies of the quantitative transplantation of mouse sarcoma. Br J Cancer 7:367-383, 1953.
7. KALLMAN RF, SILINI G, and VAN PUTTEN LM. Factors influencing the quantitative estimation of the in vivo survival of cells from solid tumors. J Natl Cancer Inst 39:539-549, 1967.
8. VAN PUTTEN LM, LELIEVELD P, and KRAM-IDSENGA LKJ. Cell-cycle specificity and therapeutic effectiveness of cytostatic agents. Cancer Chemother Rep 56:691-700, 1972.
9. HOHORST H-J, ZIEMANN A, and BROCK N. 4-Ketocyclophosphamide, a metabolite of cyclophosphamide. Arzneim Forsch 21:1254-1257, 1971.
10. STRUCK RF, KIRK MC, MELLETT LB, ET AL. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
11. TORKELSON AR, LABUDDE JA, and WEIKEL JH, JR. The metabolic fate of cyclophosphamide. Drug Metab Rev 3:131-165, 1974.
12. SCHAUMLÖFFEL E, HABERMEHL A, BROCK N, ET AL. Studies on the pharmacokinetics of cyclophosphamide in sheep. Arzneim Forsch 23:491-500, 1973.

Synthesis and Structure-Activity Relationships of Pre-Activated Analogs of Cyclophosphamide (NSC-26271)[1]

John A. Montgomery* and Robert F. Struck[2]

SUMMARY

The synthesis of approximately 80 cyclic or acyclic analogs of cyclophosphamide, isophosphamide, triphosphamide, and phosphoramide mustard is outlined briefly. All of the analogs contain at least one 2-chloroethyl or bis(2-chloroethyl)-amino group bound to phosphorus. Representatives of some of the various classes of analogs were evaluated against murine L1210 leukemia in vivo. Some of the cyclic oxidized derivatives and a phosphorodiamidic chloride yielded long-term survivors. 4-Peroxycyclophosphamide was also active against ic implanted L1210 leukemia and against two other murine tumors, Lewis lung carcinoma and C3H mammary tumor.

[Cancer Treat Rep 60:381-393, 1976]

Studies on the metabolism of cyclophosphamide (CP) (NSC-26271; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide) have shown that oxidation of C_4 of the oxazaphosphorine ring by the mixed-function oxidase of liver microsomes results in the generation of 4-hydroxycyclophosphamide (4-hydroxy-CP) (NSC-196562; 2*H*-1,3,2-oxazaphosphorin-4-ol, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide), a metabolite that is claimed by some to be the active form of CP and by others to be an unstable intermediate that yields phosphoramide mustard (NSC-69945; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-), the ultimate active form, upon spontaneous or enzyme-mediated alteration in vivo (1-5). Earlier, 4-ketocyclophosphamide (NSC-139488; 2*H*-1,3,2-oxazaphosphorin-4(3*H*)-one, 2-[bis(2-chloroethyl)amino]dihydro-, 2-oxide) and carboxyphosphamide (NSC-145124; hydracrylic acid, *N,N*-bis(2-chloroethyl)-phosphorodiamidate), urinary metabolites resulting from oxidation of 4-hydroxy-CP or aldophosphamide, its ring-opened isomer, were suggested as possible active forms of CP (6).

Because of our interest in analogs that would be equal or superior to CP as antitumor agents, we have synthesized a number of cyclic or acyclic compounds that are structurally related to CP, isophosphamide (IP) (NSC-109724; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)-amino]tetrahydro-, 2-oxide), triphosphamide (NSC-109723; 1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]-3-(2-chloroethyl)tetrahydro-, 2-oxide), or their various metabolites.

Table 1 lists the cyclic, "pre-activated" derivatives of CP, *N*-methylcyclophosphamide, and IP, all of which were synthesized by Fenton oxidation. The CP derivatives have also been prepared by van der Steen and co-workers (7) by the Fenton reaction, and the CP, *N*-methylcyclophosphamide, and IP derivatives, as well as 4-hydroperoxynorcyclophosphamide, have been prepared by Takamizawa and co-workers (2,8) by a different synthetic route.

Other cyclic structures, synthesized as shown, are listed in table 2. Included are three 4-oxo derivatives and the diastereoisomers of 4-methylcyclophosphamide (NSC-241531 and NSC-241532; 2*H*-1,3,2-oxazaphosphorine-2-amine, *N,N*-bis (2-chloroethyl)tetrahydro-4-methyl-, 2-oxide, *cis*- and *trans*-) (9,10) as well as several miscellaneous types obtained as intermediates or by-products of various reactions.

A large number of compounds that can be considered analogs of phosphoramide mustard were synthesized and are listed in table 3. All are phosphorodiamidates. Many were prepared as intermediates for the synthesis of aldophosphamide and related structures.

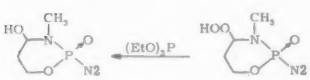
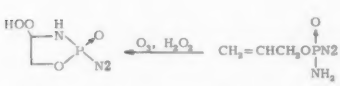
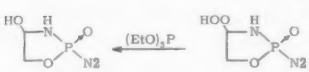
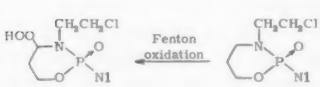
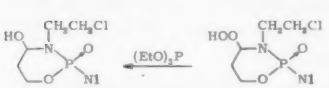
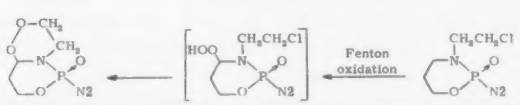
[1]Supported by contract N01-CM-43762 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala.

*Reprint requests to: Dr. John A. Montgomery, Southern Research Institute, 2000 9th Ave S, Birmingham, Ala 35205.

Table 1.—Cyclic, "pre-activated" derivatives of cyclophosphamide, N-methylcyclophosphamide, and isophosphamide

–N1 = $NHCH_2CH_2Cl$ –N2 = $N(CH_2CH_2Cl)_2$

Compound	Synthesis	Also synthesized by	Characterization
	aq KOH	Benckhuysen, et al.[a] Takamizawa, et al.[b]	IR[d], PMR[e], NMR[f], CHN[g] FDMS[h]
	Fenton oxidation	Benckhuysen, et al.[a] Takamizawa, et al.[b]	IR, PMR, NMR, CHN
	$(EtO)_3P$	Benckhuysen, et al.[a] Takamizawa, et al.[b]	IR, PMR
	Fenton oxidation	Takamizawa, et al.[b]	IR, PMR, CHN
	$(EtO)_3P$	Takamizawa, et al.[b]	IR, PMR
	O_3, H_2O_2	Takamizawa, et al.[b]	TLC[i]
	$(EtO)_3P$		TLC
	aq KOH		TLC
	Fenton oxidation	Takamizawa, et al.[c]	IR, PMR, CHN
	$(EtO)_3P$	Takamizawa, et al.[c]	IR, PMR
	aq KOH	Takamizawa, et al.[c]	IR, PMR
	Fenton oxidation		IR, PMR

[a] J Amer Chem Soc 95:7535–7537, 1973

[b] J Med Chem 18:376–383, 1975

[c] J Med Chem 17:1237–1239, 1974

[d] Infrared spectroscopy

[e] Proton magnetic resonance spectroscopy

[f] ^{13}C-Nuclear magnetic resonance spectroscopy

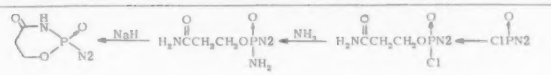
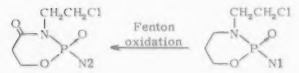
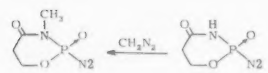
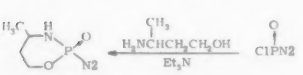
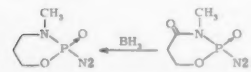
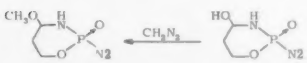
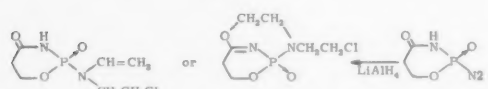
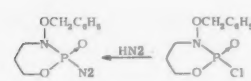
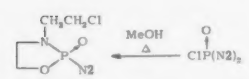
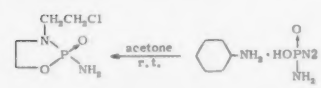
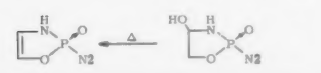
[g] Carbon, hydrogen, nitrogen elemental analysis

[h] Field desorption mass spectrometry

[i] Thin-layer chromatography

Table 2.— Miscellaneous cyclic analogs of cyclophosphamide

–N1 = –NHCH$_2$CH$_2$Cl –N2 = –N(CH$_2$CH$_2$Cl)$_2$

Compound	Synthesis	Also synthesized by	Characterization
		Arnold[a]	MS[f], IR[g], PMR[h], NMR[i], CHN[j]
		Takamizawa, et al.[b]	MS, IR, PMR, CHN
			MS, IR, PMR
 cis and trans		Arnold, et al.[c] Feil and Lamoureux[d] Thomson and Colvin[e]	MS, IR, PMR, NMR, CHN
		Arnold, et al.[c]	MS, IR, PMR
			MS
			MS
			MS
			MS
			MS, IR, PMR
			MS
			MS

[a] Second Int Symp Pharm Chem (Münster) 22, 1968

[b] J Med Chem 17:1237–1239, 1974

[c] Arzneimittel-Forsch 11:143–158, 1961

[d] Cancer Res 34:2596–2598, 1974

[e] Cancer Res 34:981–985, 1974

[f] Mass spectrometry

[g] Infrared spectroscopy

[h] Proton magnetic resonance spectroscopy

[i] ^{13}C-Nuclear magnetic resonance spectroscopy

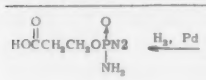
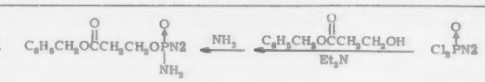
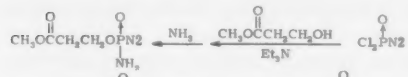
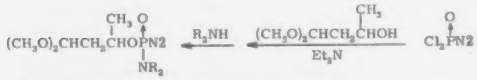
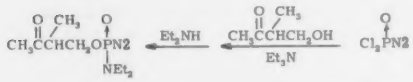
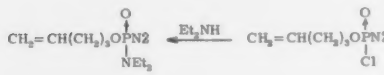
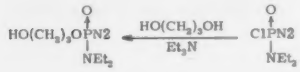
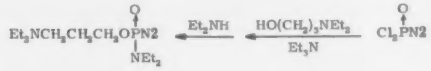
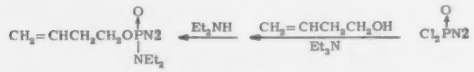
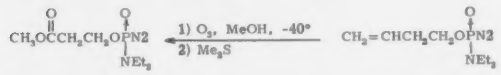
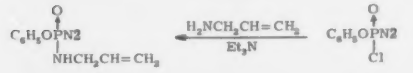
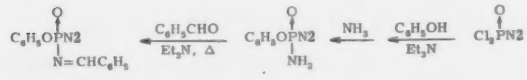
[j] Carbon, hydrogen, nitrogen elemental analysis

Table 3.—Analogs of phosphoramide mustard [N, N-bis(2-chloroethyl)phosphorodiamidates]

$-N2 = -N(CH_2CH_2Cl)_2$

Compound	Synthesis	Also synthesized by	Characterization
CH_3OPN2 (NH_2)	$\xleftarrow{NH_3}$ CH_3OPN2 (Cl) $\xleftarrow[Et_3N]{CH_3OH}$ Cl_2PN2	Colvin, et al.[a]	MS[f], IR[g], PMR[h]
CH_3OPN2 (NEt_2)	$\xleftarrow{Et_2NH}$ CH_3OPN2 (Cl)		MS, IR, PMR, CHN[i]
$Me_2NCCH_2CH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $Me_2NCCH_2CH_2OH$ $\xrightarrow[Et_3N]{}$ Cl_2PN2		MS, IR
$C_6H_5OCCH_2CH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $C_6H_5OCCH_2CH_2OH$ $\xrightarrow[Et_3N]{}$ Cl_2PN2		MS, IR
$BrCH_2CH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $BrCH_2CH_2OH$ $\xrightarrow[Et_3N]{}$ Cl_2PN2		MS, IR
$HO(CH_2)_3OPN2$ (NH_2)	$\xleftarrow{BH_3}$ $HOCCH_2CH_2OPN2$ (NH_2)		MS, IR, PMR
$HC\equiv CCH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $HC\equiv CCH_2OH$ $\xrightarrow[Et_3N]{}$ Cl_2PN2		MS, IR
$Cl(CH_2)_3OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $Cl(CH_2)_3OH$ $\xrightarrow[Et_3N]{}$ Cl_2PN2		MS, IR
$N_3(CH_2)_3OPN2$ (NH_2)	$\xleftarrow{NaN_3}$ $Cl(CH_2)_3OPN2$ (NH_2)		MS, IR
$CH_2=CHCH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $CH_2=CHCH_2OPN2$ (Cl)	Takamizawa, et al.[b]	MS, IR, PMR
$H_2NCH_2CH_2CH_2OPN2$ (NH_2)	$\xleftarrow[NH_3,\ EtOH]{H_2,\ Ni}$ $NCCH_2CH_2OPN2$ (NH_2)		MS
$NCCH_2CH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $NCCH_2CH_2OPN2$ (Cl) $\xleftarrow[Et_3N]{HOCH_2CH_2CN}$ Cl_2PN2	Takamizawa, et al.[b]	MS, IR
$CH_2=CHCH_2CH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $CH_2=CHCH_2CH_2OPN2$ (Cl)		MS, IR, PMR
$HOCH_2CHCH_2CH_2OPN2$ (OH, NH_2)	$\xleftarrow[acetone]{KMnO_4}$ $CH_2=CHCH_2CH_2OPN2$ (NH_2)		MS, IR
CH_2-CHCH_2OPN2 (O, NH_2)	$\xleftarrow{NH_3}$ CH_2-CHCH_2OH (O) $\xrightarrow[Et_3N]{}$ Cl_2PN2		MS, IR
$(EtO)_2CHCH_2CH_2OPN2$ (NH_2)	$\xleftarrow{NH_3}$ $(EtO)_2CHCH_2CH_2OH$ $\xrightarrow[Et_3N]{}$ Cl_2PN2	Takamizawa, et al.[b]	MS, IR, PMR

Table 3 (continued)

Compound	Synthesis	Also synthesized by	Characterization
		Arnold[c] Takamizawa, et al.[d]	IR, PMR
			MS, IR, PMR, CHN
			MS, IR, PMR
		Cox, et al.[e]	IR, PMR
 R = H, CH_3, C_2H_5			MS
			MS
			MS, IR, PMR, CHN
			MS, IR
			MS, IR
			MS, IR, PMR, CHN
			MS, PMR
			MS, IR
			MS
			MS

[a] Cancer Res 33:915–918, 1973

[b] J Med Chem 18:376–383, 1975

[c] Second Int Symp Pharm Chem (Münster) 22, 1968

[d] Japanese Patent 69 03,564; Chem Abstr 70:87044j, 1969

[e] Biochem Pharmacol 24:599–606, 1974

[f] Mass spectrometry

[g] Infrared spectroscopy

[h] Proton magnetic resonance spectroscopy

[i] Carbon, hydrogen, nitrogen elemental analysis

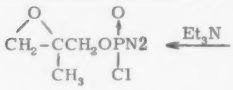
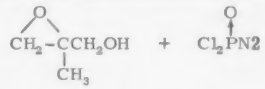
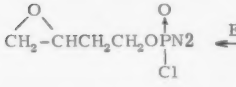
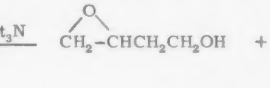
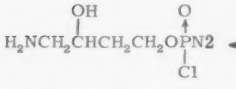
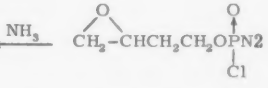
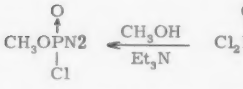
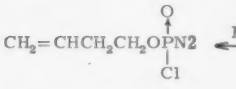
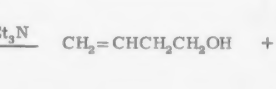
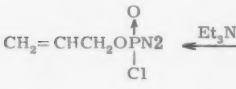
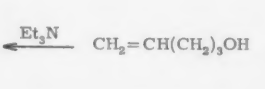
Several *N,N*-bis(2-chloroethyl)phosphoramidochloridates are listed in table 4. These were synthesized as intermediates for conversion to phosphorodiamidates.

Table 5 lists two *N,N*-bis(2-chloroethyl)-phosphoramidates, a phosphorodiamidic chloride, analogs of IP containing one, two, or three 2-chloroethylamino groups, a tris(2-chloroethyl)-phosphorodiamidic chloride, and two tetrakis(2-chloroethyl)amino derivatives. These latter structures may represent the maximum number of bis(2-chloroethyl)amino groups that can be substituted on the P→O group. We were unable to replace the chlorine in tetrakis(2-chloroethyl)phosphorodiamidic chloride (NSC-236636; phosphorodiamidic chloride, *N,N,N′,N′*-tetrakis(2-chloroethyl)-) with diethylamine, thus indicating steric hindrance to substitution by disubstituted amino groups. Replacement of chlorine by 2-chloroethylamine or other primary amines may be possible, however.

Comparison of the antitumor effect of the "preactivated" analogs, both cyclic and acyclic, against L1210 leukemia is given in table 6. All of

Table 4.— Bis(2-chloroethyl)phosphoramidochloridates

$-N2 = -N(CH_2CH_2Cl)_2$

Compound	Synthesis	Characterization
		MS[a], IR[b]
		MS
		MS
		MS, IR, PMR[c]
		MS, IR, PMR
		MS
		MS, IR

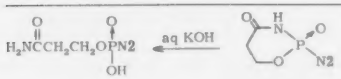
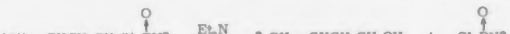
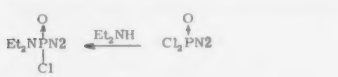
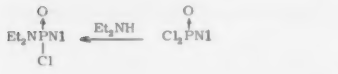
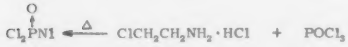
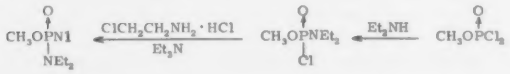
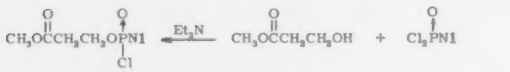
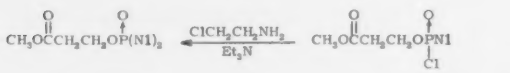
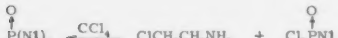
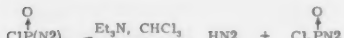
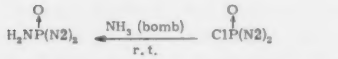
[a] Mass spectrometry

[b] Infrared spectroscopy

[c] Proton magnetic resonance spectroscopy

Table 5.—Miscellaneous phosphoramidates, phosphorodiamidates, phosphorodiamidic chlorides, phosphoramidic dichlorides, and phosphoric triamides

$-N1 = -NHCH_2CH_2Cl$ $-N2 = -N(CH_2CH_2Cl)_2$

Compound	Synthesis	Also synthesized by	Characterization
		Arnold[a]	IR[c], PMR[d], CHN[e]
			MS[f], IR
			MS, IR, PMR, CHN
			MS, IR, PMR
			MS
			IR, PMR
			MS
			MS
			MS, IR
		Takamizawa, et al.[b]	MS
			MS
			MS, IR, PMR
			MS, IR, PMR, CHN
			MS, IR, PMR, CHN

[a] Second Int Symp Pharm Chem (Münster) 22, 1968

[b] J Med Chem 17:1237–1239, 1975

[c] Infrared spectroscopy

[d] Proton magnetic resonance spectroscopy

[e] Carbon, hydrogen, nitrogen elemental analysis

[f] Mass spectrometry

Table 6.— Activity of "pre-activated" derivatives of cyclophosphamide and isophosphamide and analogs vs. L1210 leukemia

(10^5 cells, day 1 only, ip administration)

$-N2 = -N(CH_2CH_2Cl)_2$

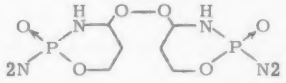
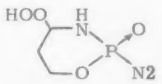
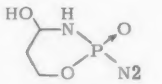
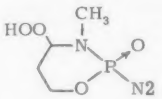
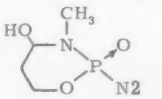
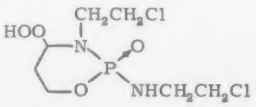
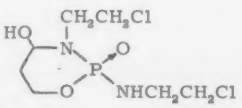
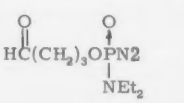
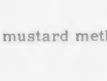
Compound	Structure	Activity at max. tol. dose (LD_{10} or less)		
		Survivors (%) (> 30 days)	ILS (%)	Max. tol. dose (mg/kg)
4-Peroxycyclophosphamide		70	167	200
4-Hydroperoxycyclophosphamide		20	81	100
4-Hydroxycyclophosphamide		30	129	100
4-Hydroperoxy-N-methylcyclophosphamide		0	-1	150
4-Hydroxy-N-methylcyclophosphamide		20	89	400
"Diethylketophosphamide"		0	15	100
4-Hydroperoxyisophosphamide		100	-	100
4-Hydroxyisophosphamide		80	184	200
Diethylhomoaldophosphamide		0	-5	200

Table 7.— Activity of latent forms of phosphoramide mustard vs. L1210 leukemia

(10^5 cells, day 1 only, ip administration)

-N2 = $-N(CH_2CH_2Cl)_2$

Compound	Structure	Activity at max. tol. dose		
		Survivors (%) (>30 days)	ILS (%)	Max. tol. dose (mg/kg)
Tetrakis(2-chloroethyl)phosphorodiamidic chloride		0	28	50
Tris(2-chloroethyl)phosphorodiamidic chloride		20	55	37.5
N,N-Bis(2-chloroethyl)-N',N'-diethylphosphorodiamidic chloride		0	8	200
N'-(2-Chloroethyl)-N,N-diethylphosphorodiamidic chloride		0	2	400
Methyl bis(2-chloroethyl)phosphoramidochloridate		0	10	75
Phosphoramide mustard methyl ester		0	21	800
Methyl N,N-bis(2-chloroethyl)-N',N'-diethylphosphorodiamidate		0	3	100

the cyclic analogs, except 4-hydroperoxy-*N*-methylcyclophosphamide (NSC-233907; hydroperoxide, 2-[bis(2-chloroethyl)amino]tetrahydro-3-methyl-2*H*-1,3,2-oxazaphosphorin-4-yl, *P*-oxide), gave some long-term survivors. Particularly impressive activity was observed for 4-peroxycyclophosphamide (4-peroxy-CP) (NSC-176986; 2*H*-1,3,2-oxazaphosphorin-2-amine, 4,4'-dioxy-bis[*N*,*N*-bis(2-chloroethyl)-tetrahydro-, 2,2'-dioxide), 4-hydroperoxyisophosphamide (NSC-207117; hydroperoxide, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-yl, *P*-oxide), and 4-hydroxyisophosphamide (NSC-208841; 2*H*-1,3,2-oxazaphosphorin-4-ol, 3-(2-chloroethyl)-2-[(2-chloroethyl)-amino]tetrahydro-, 2-oxide). The activity of 4-hydroxy-CP, 4-hydroperoxycyclophosphamide (NSC-181815; hydroperoxide, [2-[bis(2-chloroethyl)amino]-tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-yl], *P*-oxide), 4-hydroperoxyisophosphamide, and 4-hydroxyisophosphamide has also been observed by Takamizawa and co-workers (2,8). Diethylketophosphamide (NSC-236628; phosphorodiamidic acid, *N*,*N*-bis(2-chloroethyl)-*N'*,*N'*-diethyl-, 4-oxobutyl ester) was designed as an analog of aldophosphamide that would be stable enough for chemical synthesis but would degrade in vivo to an analog of phosphoramide mustard; perhaps the compound was too stable in vivo to allow for conversion to the phosphoramide mustard analog before removal from circulation. Not surprisingly, no

Table 8.— Evaluation of miscellaneous analogs of cyclophosphamide vs. L1210 leukemia

(10^5 cells, day 1 only, ip administration)

$-N2 = -N(CH_2CH_2Cl)_2$

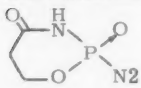
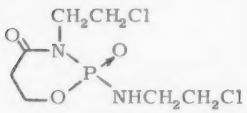
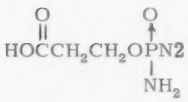
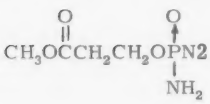
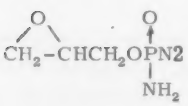
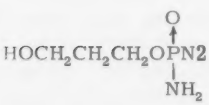
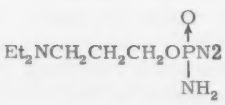
Structure	NSC No.	Max. tol. dose (mg/kg)	ILS (%)
	139488	1000	-2
	208842	400	17
	145124	500	10
	145125	300	21
	145932	200	9
	150025	300	5
	153182	300	11
	168822	200	0

Table 9.— Evaluation of cis- and trans-4-methylcyclophosphamide vs. L1210 leukemia

(10^5 cells, day 1 only, ip administration)

Compound	NSC No.	Dose (mg/kg)	ILS (%)
cis-4-Methylcyclophosphamide	241531	200	toxic
		100	46
trans-4-Methylcyclophosphamide	241532	200	toxic
		100	46
Cyclophosphamide	26271	312	197
			(80% cures)

antitumor effect was observed for diethylhomoaldophosphamide (NSC-173967; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-*N'N'*-diethyl-, 4-oxobutyl ester), which gave no indication of instability during synthesis and purification.

Table 7 compares the activity of structures we classify as latent forms of phosphoramide mustard. We expected hydrolysis of the phosphorus chlorides in vivo to the corresponding acids, thus yielding phosphorodiamidic or phosphoramidic acids related to phosphoramide mustard. Only one of the compounds, tris(2-chloroethyl)phosphorodiamidic chloride (NSC-232959), which can be considered as a latent form of triphosphoramide mustard, gave long-term survivors (20%). The methyl ester of phosphoramide mustard (NSC-178031; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-, methyl ester) was synthesized to increase lipophilicity; the results demonstrate that demethylation in vivo was too inefficient to allow for the generation of blood levels of phosphoramide mustard sufficient to produce an antitumor effect.

Other analogs that have been evaluated against L1210 leukemia but are inactive are listed in table 8. Several of these may be regarded as potentially latent forms of aldophosphamide, which could be generated by acetalase, alcohol dehydrogenase, or mixed-function oxidase action in vivo upon aldophosphamide diethyl acetal (NSC-145932; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-, 3,3-diethoxypropyl ester), alcophosphamide (NSC-153182; phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-, 3-hydroxypropyl ester), or NSC-168822 (phosphorodiamidic acid, *N,N*-bis(2-chloroethyl)-*N',N'*-diethyl-, 3-(diethylamino)propyl ester) respectively. The absence of such activity with these substrates is apparent from the test data.

Table 10. — Comparison of the activity of cyclophosphamide and 4-peroxycyclophosphamide vs. IV Lewis lung carcinoma

Implant		Treatment: ip; day 2 only		Day of death																					152nd-Day survivors/ total	Mean Life span (days)	% ILS	Median Day of death (dying)	% ILS	Drug toxicity control 152nd-day survivors/total
Cells	Route	NSC No.	Name	Dosage (mg/kg/dose)	3	15	16	17	18	19	20	21	22	23	24	25	27	29	41	42	50	51	52	66	79	89	92	97	106	121					
10^6	IV	Control (untreated)						1		1	2	3	1																		0/10	18.7		19.0		
		26271	Cyclophosphamide	312	1																										9/10	>138.0	>+637	3.0	-85	8/10
				156									1											1		1				7/10	>126.9	>+578	19.0	+315	9/9
				78											1					1		1			1					5/10	>107.5	>+474	50.0	+163	10/10
		176986	4-Peroxycyclophosphamide	268															1		1	1	1		1				1		4/10	>103.6	>+454	59.0	+210	10/10
				134									2	2	1	3	1	1												0/10	24.5	+31	24.5	+28	10/10
				67			1	1	1		3	2	1		1															0/10	19.6	+4	20.0	+5	10/10

Table 11. — Chemotherapy of IV implanted C3H mammary tumor cells with 4-peroxycyclophosphamide and cyclophosphamide

| Implant | | Treatment | | | Day of death | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 55th Day survivors/ total | Toxicity control survivors (%) | Toxicity deaths in tumor-bearing animals | Median day of death (dying) | Range | % ILS |
|---|---|---

Table 12. — Study to determine the antileukemic activity of 4-peroxycyclophosphamide against intracerebrally implanted L1210 leukemia

Implant Route	Implant Cells	Agent	Dosage (mg/kg/dose)	48th-Day survivors/ total	Median % ILS	Approx. No. cells surv. at end of therapy	Drug toxicity survivors/ total
IC	10^5	Cyclophosphamide (CPA)	300	0/10	+37	5.6×10^4	10/10
			200	0/10	+25	2.4×10^4	9/9
		4-Peroxycyclophosphamide (4-Peroxy CPA)	400	0/10	--	--	1/10
			300	0/10	+100	3.2×10^2	10/10
			200	0/10	+50	1.0×10^5	10/10
			133	0/10	+25	2.4×10^4	10/10
			89	0/10	+12	1.0×10^5	10/10
IC	10^4	CPA	300	0/10	+40	1.3×10^2	10/10
			200	0/10	+20	2.4×10^3	9/9
		4-Peroxy CPA	400	0/10	--	--	1/10
			300	0/10	+85	1.0×10^0	10/10
			200	0/10	+40	1.3×10^2	10/10
			133	0/10	+30	5.6×10^2	10/10
			89	0/10	+10	1.0×10^4	10/10

6. Struck RF, Kirk MC, Mellett LB, et al. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
7. van der Steen J, Timmer EC, Westra JG, et al. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
8. Takamizawa A, Matsumoto S, Iwata T, et al. Studies on cyclophosphamide metabolites and their related compounds. 2. Preparation of an active species of cyclophosphamide and related compounds. J Med Chem 18:376-383, 1975.
9. Arnold H, Bourseaux F, and Brock N. Relations between chemical structure and cancerotoxic activity in phosphamide bis(2-chloroethyl)aminoesters. Arzneim Forsch 11:143-158, 1961.
10. Feil VJ, and Lamoureux CJH. Alopecia activity of cyclophosphamide metabolites and related compounds in sheep. Cancer Res 34:2596-2598, 1974.

Importance of Pharmacokinetic Studies on Cyclophosphamide (NSC-26271) in Understanding its Cytotoxic Effect[1,2]

M. G. Donelli,* I. Bartošek, A. Guaitani, A. Martini, T. Colombo, M. A. Pacciarini, and R. Modica[3,4]

SUMMARY

Pharmacokinetic studies on cyclophosphamide (CP) and its alkylating metabolites produced by hepatic biotransformation have been performed in vivo in animals and in vitro in the perfused liver. CP levels were determined by a gas-chromatographic method combined with mass-spectrometry, and production of alkylating metabolites was assayed by the 4-(4-nitrobenzyl)pyridine reaction for alkylating compounds. Differences in serum drug levels between normal rats and rats bearing Walker 256 carcinosarcoma were observed in vivo and were confirmed by the liver-perfusion technique. Pharmacokinetic parameters and enzyme kinetic data both in normal and in tumor-bearing animals will be presented. The disappearance of CP and the corresponding formation of CP metabolites was significantly modified when (*a*) CP was given after previous treatment with a compound which alters its biotransformation (ie, phenobarbital, an inducer of microsomal metabolism), (*b*) CP was given after previous treatment with CP (which inhibits microsomal metabolism), or (*c*) CP was given with competitive substrates of aldehyde oxidase or dehydrogenase (glyceraldehyde, chloral hydrate, and disulfiram). Results obtained in animals or in the perfused liver will be discussed. The significance of this modified CP metabolism in influencing its cytotoxic effect will be discussed and correlations between drug levels and activity will be presented.

[Cancer Treat Rep 60:395-401, 1976]

The use and effectiveness of a chemical agent in the treatment of malignant diseases is partly governed by the pharmacologic disposition of the drug at the whole-body and cellular levels, which is related to the effective concentration of the agent at the receptor site and therefore influences the drug's duration and intensity of action. Pharmacokinetic studies on cyclophosphamide (CP) and its alkylating metabolites produced by hepatic biotransformation have been undertaken by our group, either in vivo in experimental animals or in vitro in the perfused liver, to obtain information which may be useful in understanding the cytotoxic effect of CP. The amount of the alkylating metabolites was assayed by the 4-(4-nitrobenzyl)-pyridine (NBP) reaction for alkylating compounds (1) in order to obtain an estimate of the metabolite production. Although the NBP reaction only allows a rough estimate of the metabolites and does not discriminate between cytotoxic and noncytotoxic compounds, this technique may still be useful if the production of NBP metabolites is compared to the disappearance rate of unchanged CP. The latter can now be determined in biologic specimens by a highly sensitive and specific gas-chromatographic technique combined with mass spectrometry (2).

[1]Supported by contracts 088-72-I-BIAC from Euratom and 72,01081,04 from Consiglio Nazionale delle Ricerche.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy.

[4]We wish to thank Prof. Norbert Brock (Asta-Werke AG, Brackwede, West Germany) and the Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md, for the generous supply of cyclophosphamide and isophosphamide (used as a standard for the gas-chromatographic method). The excellent technical assistance of Mrs. S. Villa, Mr. M. Broggini, and Miss M. Mainini is gratefully acknowledged.

**Reprint requests to:* M. G. Donelli, MD, Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea 62, 20157 Milan, Italy.

INFLUENCE OF THE PRESENCE OF A TUMOR ON CP METABOLISM

In the pharmacokinetic studies on CP, the first aspect investigated was the influence of a tumor on the distribution and metabolism of this compound. It is well known that tumors can markedly affect the metabolic capacity of the host, resulting in modifications in the metabolism, distribution, and pharmacologic activity of administered compounds (3,4). One should therefore be cautious in extrapolating results from the normal condition to the pathologic condition, the one of real clinical interest. Figure 1 shows the disappearance curve of unchanged CP from the serum of CD rats bearing sc Walker 256 carcinosarcoma after iv injection of 50 mg/kg. At all times tested it is evident that CP levels were higher in tumor-bearing rats compared to controls. The relative production of the NBP-positive metabolites was lower in the presence of the Walker 256 tumor, as shown by the levels of alkylating activity in the serum (fig 2). The corresponding pharmacokinetic parameters, reported in table 1, are in good agreement with these findings; the half-life of CP was longer and the rate of metabolite production was lower in the presence of the Walker 256 tumor.

TABLE 1.—Serum pharmacokinetic parameters of CP (50 mg/kg iv) and its alkylating metabolites in CD rats bearing Walker 256 carcinosarcoma and in controls

Kinetic parameters*	Controls	Rats bearing Walker 256 carcinosarcoma
CP		
Peak serum level (μg/ml $\pm$ SE)	239 $\pm$ 31 (1)†	212 $\pm$ 15 (1)†
Half-life (min)	32	50
Vd (liter/kg)	0.38	0.36
C × t (μg min/ml)	7427 $\pm$ 928	9521 $\pm$ 936
Drug clearance (liter/min/kg)	0.01	0.008
NBP metabolites		
Peak serum level (nmols Eq HN_2/ml $\pm$ SE)	131 $\pm$ 12 (20)†	82 $\pm$ 13 (40)†
Half-life (min)	28	47
K_f (nmols Eq HN_2/ml/min)	0.07	0.03
C × t (nmols Eq HN_2 × min/ml)	9346	9855

*Vd = volume of distribution; C × t = area under the concentration vs time curve; K_f = rate of formation of NBP-positive metabolites.

†Nos. in parentheses = time in min at which the peak is reached.

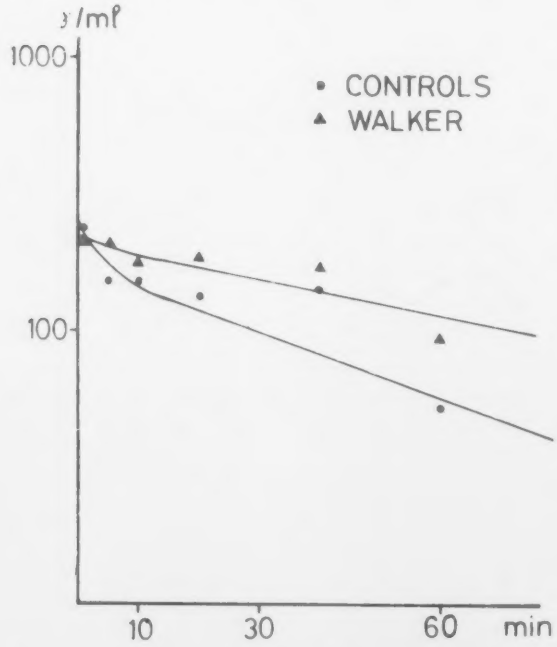


FIGURE 1.—Serum levels of CP (50 mg/kg iv) in rats bearing sc Walker 256 carcinosarcoma compared to controls.

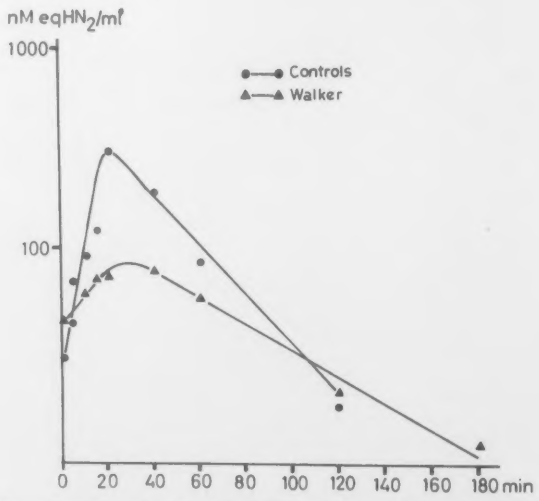


FIGURE 2.—Serum levels of the NBP-positive metabolites after an iv injection of 50 mg/kg of CP in rats bearing sc Walker 256 carcinosarcoma compared to controls.

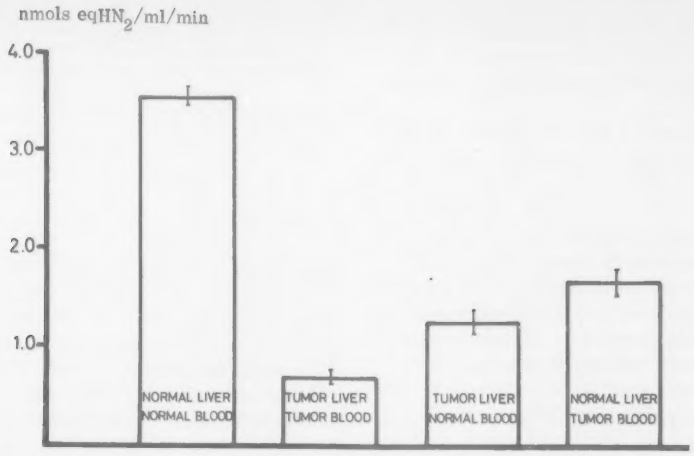


FIGURE 3.—Metabolite formation rate during perfusion of liver (initial CP concentration = 150 μg/ml of medium) from rats bearing sc Walker 256 carcinosarcoma.

Similar results have been obtained by perfusing the liver of CD rats, both normal and tumor-bearing, with CP (150 μg/ml) according to the technique described by Bartošek et al (5). The production rate of CP metabolites (*see* fig 3) by livers obtained from tumor-bearing rats perfused with tumor blood was about 20% of control livers perfused with normal blood. It is interesting to observe that cross experiments revealed the presence of an inhibitor in the blood of the tumor-bearing animals which decreased CP hepatic metabolism. In order to better characterize these metabolic differences between normal and tumorous liver, the overall affinity constants and maximal rates of CP disappearance and/or of formation of its alkylating metabolites were measured in the model of liver perfusion (table 2). The data confirmed the marked alteration of the metabolic capacity of the liver induced by the growth of an extrahepatic tumor.

INFLUENCE OF COMPOUNDS INTERACTING WITH CP METABOLISM

With the knowledge that CP is first activated and then inactivated by hepatic enzymes, and with the wealth of information available on the stimulation and inhibition of enzymatic activities, it was considered of interest to study the possibility of manipulating CP metabolism by enhancing or inhibiting the activity of the enzymatic systems responsible for the different metabolic steps. However, the possibility of affecting the cytotoxic

TABLE 2.—Affinity constants (K_{aff}) and maximum rates (V_{max}) of CP transformation in perfused liver isolated from normal and Walker 256 carcinosarcoma-bearing male rats*

	K_{aff} (mM)		V_{max} (nmol/ml/min)	
Animals	CP disappearance	Metabolite formation	CP disappearance	Metabolite formation
Normal	4.3 $r = 0.76$	33.2 $r = 0.97$	98.7 $r = 0.76$	171.8 $r = 0.97$
Walker 256 tumor-bearing	1.4 $r = 0.82$	3.2 $r = 0.73$	4.3 $r = 0.82$	14.3 $r = 0.73$

*K_{aff} and V_{max} were calculated using the integrated form of the Michaelis-Menten equation:

$$\frac{1}{t} \ln \frac{S_o}{S_t} = -\frac{1}{K_{aff}} \cdot \frac{S_o - S_t}{t} + \frac{V_{max}}{K_{aff}}.$$

r = Correlation coefficient of the linear regression. Initial CP concentrations in the perfusion medium ranged from 0.1915 to 1.915 mM.

activity of CP by modifying its metabolism is limited by the fact that the final response is the result of a complex balance between active and inactive metabolites.

Our objective concerned the possibility of modifying CP activity by the concomitant administration of compounds (table 3) interacting with some metabolic steps of CP metabolism. According to the metabolic pathway proposed by Connors et al (6), previous treatment with phenobarbital (PB) or CP should affect the mixed-function oxidase system responsible for the activation of CP, whereas glyceraldehyde, chloral hydrate, and disulfiram should inhibit a step of the inactivation process.

Interaction Between PB and CP

The first compound investigated was PB, a well-known inducer of microsomal enzymes (7), which is widely used in supportive therapy of cancer patients. PB given at a dose of 80 mg/kg ip 48 and 24 hours before treatment with CP has been shown to be highly effective in stimulating the activity of the microsomal enzymes.

The concentrations of unmetabolized CP and its NBP-positive metabolites were measured in vivo in the serum and urine of PB-pretreated CD rats and CD_1 mice and in vitro in the perfusion medium of livers isolated from pretreated rats. The results obtained (8) indicated accelerated metabolism of CP with an initial stimulation of the activation process. Table 4 shows the pharmacokinetic parameters of CP in control animals and PB-pretreated mice. The faster disappearance of CP was accompanied by a higher rate of metabolite production, followed by their more rapid disappearance when PB was given. These findings on the metabolism of CP when combined with PB correlate with the modified biologic activity in the same experimental conditions.

TABLE 3.—Compounds investigated in the present study and their postulated site of interaction in CP metabolism

Compound	Postulated site of interaction
PB	Inducer of microsomal enzymes and aldehyde oxidase
CP	Inhibitor of microsomal enzymes
Glyceraldehyde	Competitive substrate of aldehyde oxidase and/or dehydrogenase
Chloral hydrate	Competitive substrate of aldehyde oxidase and/or dehydrogenase
Disulfiram	Inhibitor of aldehyde dehydrogenase

TABLE 4.—Serum kinetic parameters of CP (50 mg/kg iv) and its alkylating metabolites after administration of CP to CD_1 mice pretreated with saline or PB (80 mg/kg ip) (48 and 24 hrs before CP)*

Kinetic parameters	CP	PB + CP
CP		
Peak serum level (μg/ml ± SE)	58 ± 3 (1)†	39 ± 3 (1)†
Half-life (min)	5	3
C × t (μg × min/ml)	430	201
Drug clearance (liter/min/kg)	0.11	0.25
NBP metabolites		
Peak serum level (nmols HN_2/ml)	111 ± 9 (15)†	106 ± 7 (10)†
Half-life (min)	18	10
K_f (nmols HN_2/ml/min)	0.17	0.58

*See table 1 for abbreviations.

†Nos. in parentheses = time in min at which the peak is reached.

Table 5 reports the cytotoxicity in mice of CP associated with PB expressed as antitumor or immunosuppressive activity. The antitumor activity was evaluated by recording the survival time of BDF_1 mice inoculated ip with L1210 leukemia cells and treated with CP and PB or by counting the number of leukemic cells 5 days after CP treatment. The immunosuppressive activity was measured by counting the number of hemolytic plaque-forming cells (PFC) in the spleens of BDF_1 mice 4 days after the ip injection of sheep erythrocytes using the technique of Jerne et al (9). The marked cytotoxic activity exerted by CP is partly antagonized by the CP-PB interaction (table 5).

Effect of Previous Treatment With CP on its own Metabolism

Another interaction of possible clinical relevance concerns the influence of previous treatment with CP on its own metabolism. Since cytotoxic agents have been shown to inhibit the microsomal enzyme activity of the liver (10,11), it was postulated that CP metabolism could be impaired at the microsomal level. Normal or tumor-bearing (sc Walker 256 carcinosarcoma) CD rats were treated with a single injection of 120 mg/kg of CP ip or fractionated injections of the same total amount (20 mg/kg ip daily × 6 days). These two treatment schedules have a proven efficacy in producing a complete disappearance of the Walker 256 tumor. Seven days after the start of treatment, the liver was

TABLE 5.—Effect of PB pretreatment on the immunosuppressive and antileukemic activity of CP in $BD2F_1$ mice

PB (mg/kg ip)	Days	CP (mg/kg ip)	Days	PFC/10^6 spleen cells	No. of L1210 cells in ascites (Day 5)	MST (days)
80 × 2	0,1	—	—	683	453 × 10^6	7.9
—	—	25-100	2	29	41 × 10^6	19.6
80 × 2	0,1	25-100	2	181*	205* × 10^6	14.9*

*Data summarized from Garattini et al (8). The values of controls were the same as for the PB-pretreated mice.

isolated and perfused with a medium containing 150 μg/ml of CP. Table 6 shows the rate constants of CP disappearance and of NBP metabolite formation in tumor-bearing rats compared to controls. It can be seen that previous treatment with CP reduced both the disappearance rate of CP and the metabolite production in normal rats; however, the same treatment in tumor-bearing rats, leading to the complete disappearance of the tumor, seemed to repair the metabolic conditions of the liver. These data confirm that more prolonged courses with a compound may alter its metabolism in the subsequent administration.

Interaction Between Glyceraldehyde, Chloral Hydrate, or Disulfiram and CP

Another type of interaction investigated leads to an increased cytotoxic activity of CP by increasing the duration in the body of the active compound aldophosphamide. The inhibition of aldehyde oxidase and/or dehydrogenase by means of competitive substrates or inhibitors of these enzymes such as glyceraldehyde, chloral hydrate, or disulfiram should result in a reduced inactivation of aldophosphamide.

C57BL mice bearing 25-day-old im Lewis lung carcinomas and $(C3H \times O2O)F_1$ mice bearing spontaneous mammary carcinomas (about 5 g) pretreated with one of these compounds were given an iv injection of CP (80 mg/kg) and the serum levels of CP and its metabolites were measured. Table 7 reports the serum pharmacokinetic parameters in two of these combinations, where the differences are more evident, as an example of the change induced in CP metabolism during this kind of interaction. The half-life of CP was prolonged, ie, the drug persisted longer in the serum; that this may be interpreted as an expression of a reduced metabolism is confirmed by observing the metabolite production, which showed a reduced peak. However, the alkylating metabolites disappeared from the blood at a slower rate than in controls, as shown by the longer half-life and the higher concentration × time value.

The inhibition of the inactivation process resulted in a longer persistence of CP metabolites in the body, which could account for the increased cytotoxic activity of CP when combined with glyceraldehyde, chloral hydrate, or disulfiram. Indeed, when the effect of these combinations was studied in terms of antitumor activity on the same experi-

TABLE 6.—Effect of previous CP treatment on its own metabolism in isolated perfused rat livers

Liver and blood from—	Treatment	Mean nmol/ml/min ± SE: CP disappearance rate*	Mean nmol/ml/min ± SE: Metabolite formation rate*
Normal rats	Control	5.09 ± 1.19‡	0.849 ± 0.159‡
	CP (1 × 120 mg/kg)†	2.46 ± 0.27	0.554 ± 0.143
	CP (6 × 20 mg/kg)	3.30 ± 0.10	0.367 ± 0.039
Walker 256 tumor-bearing rats	Control	1.10 ± 0.03§	0.040 ± 0.007¶
	CP (1 × 120 mg/kg)	2.93 ± 0.08	0.327 ± 0.078
	CP (6 × 20 mg/kg)	4.78 ± 1.90**	0.210 ± 0.024

*Disappearance rate of CP and metabolite formation rate were calculated from the integrated form of the Michaelis-Menten equation. Initial CP concentration in the perfusion medium was 574.5 μM (150 μg/ml).

†CP was administered ip either in 1 dose of 120 mg/kg 7 days before liver perfusion or in fractionated injections of 20 mg/kg for 6 consecutive days.

‡Mean of 4 perfusions.

§$P < 0.05$ vs normal perfusions.

¶$P < 0.01$ vs normal perfusions.

**Mean of 3 perfusions.

TABLE 7.—Effect of disulfiram and chloral hydrate pretreatment on the serum kinetic parameters of CP and its alkylating metabolites (80 mg/kg iv) in C57BL mice bearing im Lewis lung carcinoma (25 days) or $(C3H \times O2O)F_1$ mice bearing mammary carcinoma (about 5 g)*

Kinetic parameters	CP†	CP + disulfiram†‡	CP§	CP + chloral hydrate‡§
CP				
Peak serum level (μg/ml ± SE)	155 ± 35	125 ±27	129 ± 12	125 ± 18
Half-life (min)	13	18	5	8
Vd (liter/kg)	0.58	0.46	0.59	0.67
C × t (μg × min/ml)	1639 ± 239	1450 ± 326	1012 ± 120	1152 ± 112
NBP metabolites				
Drug clearance (liter/min/kg)	0.048	0.055	0.079	0.069
Peak serum level (nmols Eq HN_2/ml ± SE)	178 ± 21	134 ± 18	153 ± 9	70 ± 18
Half-life (min)	34	50	19	38
K_t (nmols Eq HN_2/ml/min)	0.07	0.07	0.06	0.07
C × t (nmols Eq HN_2 × min/ml)	6590	7960	1973	3052

*CP was injected in a single dose for Lewis lung carcinoma and in 3 doses (Days 0, 2, and 4) for mammary carcinoma. See table 1 for abbreviations.

†C57BL mice.

‡Disulfiram (80 mg/kg ip) was administered 24 hrs before CP and chloral hydrate (10 mg/kg ip) was administered 15 mins before each treatment of CP.

§$(C3H \times O2O)F_1$ mice.

mental tumor systems, the results obtained indicated a marked increase in therapeutic efficacy when an inhibitor of aldehyde oxidase or dehydrogenase was given with CP.

Table 8 shows the effect of this interaction on the mammary carcinoma of $(C3H \times O2O)F_1$ mice. Tumor diameters were measured using calipers on coded animals; values given in millimeters were then corrected to weight using the formula of the spheroid, $W = ab^2/2$, where a = the length and b = the width of the tumor, assuming unit density. There was a linear relationship between tumor weight and time after the start of treatment. The student's t test applied to the rate of growth of the tumor always resulted in significant differences between the CP-treated mice and the group receiving a previous treatment with one of these agents.

The effect of these combinations on the Lewis lung carcinoma is reported in table 9. When associated with a slightly effective dose of CP, disulfiram and chloral hydrate, which by themselves show no activity on growth or on metastases, produced a marked increase in the antitumor activity of the cytotoxic agent. However, the combination of glyceraldehyde and CP seemed not effective in the case of the Lewis lung carcinoma.

TABLE 8.—Effect of CP on the growth of mammary carcinoma in $(C3H \times O2O)F_1$ mice pretreated with disulfiram, chloral hydrate, or glyceraldehyde

	Tumor weight† increase (g/day)	
Agent*	Agent alone	Agent + CP
None	0.187	0.103
Disulfiram	0.360	0.042
Chloral hydrate	0.234	0.032
Glyceraldehyde	0.214	0.024

* Chloral hydrate (10 mg/kg ip) or glyceraldehyde (8 mg/kg ip) were administered 15 mins before CP (80 mg/kg iv) on Days 1, 3, 10, 12, 15, and 18, whereas disulfiram (10 mg/kg ip) was administered 24 hrs before each treatment.

†This value was obtained by calculating the slope (b) of the equation of the curve ($y = a + bx$). Tumor weight at start of treatment = 1.8 g.

CONCLUSIONS

The studies described in this paper, particularly in connection with a possible increase in CP activity, need further investigation to assess the toxicologic parameters as well as the activity on other experimental tumors. The therapeutic indexes so derived may permit evaluation of interactions between CP and the inhibitors studied. However, these findings, although certainly only suggestive and limited to our experimental conditions, indicate that the possibility of manipulating CP metabolites and therefore modifying its activity should not be discarded.

It must be emphasized that there is some question as to the meaning of the serum levels of CP metabolites insofar as the methods used for their

TABLE 9.—Antitumor and antimetastatic activity of CP in C57BL mice bearing im Lewis lung carcinoma pretreated with disulfiram, chloral hydrate, or glyceraldehyde

Drug	Treatment (mg/kg)	Tumor weight* (g)	Metastases at death		MST (days)
			No.	Weight (mg ± SE)	
Controls		7.8 ± 0.4	23 ± 3	117 ± 33	29 ± 2
CP†		6.3 ± 0.3‡	21 ± 2	104 ± 20	40 ± 4‡
CP† + disulfiram	80 ip 24 hrs before CP	4.3 ± 0.4§	11 ± 3§	41 ± 14¶	36 ± 3‡
CP† + chloral hydrate	80 ip 15 mins before CP	5.0 ± 0.5¶	9 ± 4§	17 ± 7§	38 ± 2‡
CP† + glyceraldehyde	30 ip 15 mins before CP	4.5 ± 0.4§	30 ± 3	194 ± 12	40 ± 3‡
Disulfiram		7.4 ± 3.8	19 ± 5	95 ± 33	31 ± 2
Chloral hydrate		7.6 ± 1.0	23 ± 5	106 ± 27	32 ± 1
Glyceraldehyde		7.9 ± 0.5	25 ± 4	164 ± 60	30 ± 1

*On day of sacrifice (Day 25).
†80 mg/kg iv on Day 14.
‡$P < 0.01$ relative to controls.
§$P < 0.01$ relative to CP.
¶$P < 0.05$ relative to CP.

quantitative determination are insufficiently specific. Only the separate measurement of the various CP metabolites possessing different cytotoxic activities would determine what role is played by the relative rates of production of such metabolites.

In any case, the complete change in the antitumor activity of CP obtained as a result of the interaction with other agents (an antagonist in the case of PB and a potentiator in the case of disulfiram, chloral hydrate, and glyceraldehyde) points out the intricacies of this field and accordingly the caution that should be exerted in combining drugs with CP at the clinical level.

REFERENCES

1. FRIEDMAN OM, and BOGER E. Colorimetric estimation of nitrogen mustards in aqueous media. Hydrolytic behavior of bis-(2-chloroethyl)amine, *nor*-HN2. Anal Chem 33:906-910, 1961.
2. PANTAROTTO C, BOSSI A, BELVEDERE G, ET AL. Quantitative GLC determination of cyclophosphamide and isophosphamide in biological specimens. J Pharm Sci 63:1554-1558, 1974.
3. ROSSO R, DONELLI MG, FRANCHI G, ET AL. Impairment of drug metabolism in tumor-bearing animals. Eur J Cancer 7:565-577, 1971.
4. KATO R, TAKANAKA A, TAKAHASHI A, ET AL. Drug metabolism in tumor-bearing rats. I. Activities of NADPH-linked electron transport and drug-metabolizing enzyme systems in liver microsomes of tumor-bearing rats. Jap J Pharmacol 18:224-244, 1968.
5. BARTOŠEK I, GUAITANI A, and GARATTINI S. Long-term perfusion of isolated rat liver. Pharmacology 8:244-258, 1972.
6. CONNORS TA, COX PJ, FARMER PB, ET AL. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
7. CONNEY AH. Pharmacological implications of microsomal enzyme induction. Pharmacol Rev 19:317-366, 1967.
8. GARATTINI S, BARTOŠEK I, DONELLI MG, ET AL. Interactions of anticancer agents with other drugs. *In* Pharmacological Basis of Cancer Chemotherapy. Baltimore, Williams and Wilkins, 1975, pp 565-593.
9. JERNE NK, and NORDIN AA. Plaque formation in agar by single antibody producing cells. Science 140:405, 1963.
10. DONELLI MG, FRANCHI G, and ROSSO R. The effect of cytotoxic agents on drug metabolism. Eur J Cancer 6:125-126, 1970.
11. TARDIFF RG, and DUBOIS KP. Inhibition of hepatic microsomal enzymes by alkylating agents. Arch Int Pharmacodyn Ther 177:445-456, 1969.

Approaches to the Pharmacokinetics of Cyclophosphamide (NSC-26271): Quantitation of Metabolites[1,2]

Ian Jardine, Robert Brundrett, Michael Colvin,* and Catherine Fenselau[3,4]

SUMMARY

Recent evidence has implicated phosphoramide mustard as a highly active metabolite of cyclophosphamide (CP). This compound has been shown to be produced from CP both in vitro and in vivo. To further study the role of this active metabolite we have devised methods to quantitate the compound in human serum and urine using gas chromatography-chemical ionization mass spectrometry. Tetradeuterated phosphoramide mustard has been synthesized and is used as an internal standard. CP and *nor*-nitrogen mustard have also been quantitated in human samples using analogous tetradeuterated internal standards.

[Cancer Treat Rep 60:403-408, 1976]

In an attempt to quantitate cyclophosphamide (CP) and its metabolites with a view to ultimately optimizing the clinical regimen of the drug through a better understanding of the pharmacokinetics involved, we have developed a procedure which is general for all major CP metabolites. The procedure is also selective for each metabolite and is highly sensitive in order to quantitate less abundant metabolites and to quantitate metabolites from small fluid or tissue samples.

Gas chromatography-mass spectrometry (GC-MS) is a highly specific and sensitive analytic technique (1). Sensitivity is particularly impressive when the selected ion monitoring (SIM) technique is used (1). In this technique specific ions from the compound of interest are continually monitored and since sample is not wasted as when the mass spectrometer scans across the whole mass range, a considerable increase in sensitivity can be achieved. Another recent advance in MS has been the introduction of chemical ionization (CI) (2). In this technique mass spectra are produced by ionizing a reagent gas in the mass spectrometer source at the relatively high pressure of 1 torr (1 mm of Hg) and by allowing the ions formed to interact with sample vapor introduced, for example, from a gas chromatograph. The resulting sample ions are then analyzed by the mass spectrometer. For example, upon electron impact (EI) at high source pressure, isobutane (iso-C_4H_{10}) reagent gas produces tert-$C_4H_9^+$ (m/e 57) ions as the major species. When a sample (M) is introduced, a proton is transferred from the abundant tert-$C_4H_9^+$ ions to produce abundant $(M + H)^+$ ions with very little fragmentation:

$$C_4H_9^+ + M \longrightarrow MH^+ + C_4H_8$$

This process is particularly useful for the analysis of drugs and metabolites since they often contain an accessible basic site to which the proton may readily attach. The sensitivity resulting from this method of ionization is comparable to that of EI ionization (3) and can often be better when using SIM since almost all of the sample ion current produced in CI will be in one ion, $(M + H)^+$, which can be selectively monitored.

In order to effect quantitation of any compound, especially if it is extracted from biologic sources,

[1]Supported by Public Health Service grants CA-16783 (National Cancer Institute) and GM-21248 (National Institutes of General Medical Sciences), and Research Career Development Awards CA-00103 (National Cancer Institute; Dr. Colvin) and GM-70417 (National Institutes of General Medical Sciences; Dr. Fenselau) from the National Institutes of Health, Department of Health, Education, and Welfare; and by grants from the Andrew Mellon Fund and the American Cancer Society, Maryland Division.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Department of Pharmacology and Experimental Therapeutics (Drs. Jardine and Fenselau) and Oncology Division, Department of Medicine (Drs. Brundrett and Colvin), The Johns Hopkins University School of Medicine, Baltimore, Md.

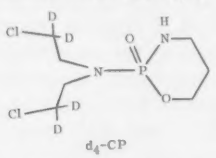
[4]We would like to thank Mr. M. L. Appler and Mrs. M. N. Kan for their technical assistance.

*Reprint requests to: Dr. Michael Colvin, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md 21205.

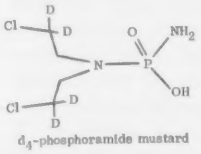
it is necessary to have an internal standard which can be added at the beginning of an experiment and which will account for subsequent losses of material. The best internal standard for mass spectrometric quantitation has been found to be stable isotopic analogs of the compound under study since these analogs will act identically under all chemical and physical processes, for example, extraction, derivatization, gas chromatographic retention, and mass spectrometric ionization. With this in mind we synthesized a tetradeuterated analog of *nor*-nitrogen mustard from which we could synthesize appropriate standards for any of the *N,N*-bis(2-chloroethyl) metabolites of CP (or indeed, any similar alkylating agent, eg, chlorambucil, aniline mustard etc). This was accomplished as shown in figure 1.

This synthesis is particularly useful since the resulting deuterium incorporation is very high, reflecting the high isotopic purity of lithium aluminum deuteride. The isobutane CI spectrum of d_4-*nor*-nitrogen mustard derivatized with trifluoroacetic anhydride (TFA) (see below) is shown in figure 2 compared to the derivatized undeuterated material. The high deuterium incorporation is readily ascertained. It should be noted that although these spectra are only plotted from m/e 200 to m/e 280, there are no other ions in the spectra except those attributable to the isobutane gas, for example, at m/e 57.

Internal standards for the other two compounds in which we are particularly interested, CP and phosphoramide mustard, a compound shown to be produced from CP both in vitro (4) and in vivo (5), were synthesized from d_4-*nor*-nitrogen mustard by published methods (6,7).



d_4-CP



d_4-phosphoramide mustard

It should be pointed out that these deuterated compounds would not be as useful for EI ionization-MS since under EI the compounds would fragment readily at the bond α to the mustard nitrogen and two of the deuteriums would be eliminated (4).

It is possible to quantitate materials simply by using the probe inlet of a mass spectrometer (8). However, this is generally not as sensitive a method as gas chromatographic introduction and the gas chromatograph, of course, separates the material from a mixture, which is often critical in biologically derived samples. However, it is often necessary to derivatize material before introduction

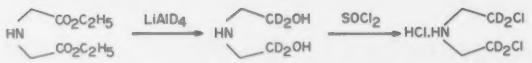


FIGURE 1.—Synthesis of d_4-*nor*-nitrogen mustard (HN).

to a gas chromatograph. A suitable derivative for *nor*-nitrogen mustard is the trifluoroacetylated compound obtained by reaction of the *nor*-nitrogen mustard in ethylacetate with TFA. The appropriate gas chromatographic and CI (isobutane) spectra of the derivatized compound and the d_4 compound are shown in figure 2. To illustrate the necessity of derivatization and also to indicate the sort of sensitivity obtainable with the technique of SIM, figure 3 shows the $(M + H)^+$ ion (m/e 142) for underivatized *nor*-nitrogen mustard at 100 ng injected on column compared to the $(M + H)^+$ ion (m/e 238) for the *nor*-nitrogen mustard-TFA at 1 ng and 100 pg injected on column. Considerable tailing is evident for the underivatized *nor*-nitrogen mustard, while, with derivatization, low picogram levels are easily measurable with little peak tailing. It should be noted that there is no quantitative relationship between these three recordings since each was measured at different attenuation and gain.

Figure 4 shows the gas chromatogram and CI mass spectra of derivatized (TFA) CP and d_4-CP. The derivatization was by the method of Pantarotto et al (9).

Figure 5 shows the gas chromatographic trace of phosphoramide mustard after derivatization with ethereal diazomethane (4). As has been pointed out previously (4), three products were obtained: the monomethylated product (last to elute from the gas chromatogram), the dimethylated product (center gas chromatographic peak), and the trimethylated product (first to elute from the gas chromatogram). Although from this gas chromatographic trace it may appear that there is more dimethylated product, in fact, as can be readily ascertained from a direct insertion probe mass spectrum of the mixture, there is more monomethylated product produced. However, substantial losses of this material occur in the gas chromatogram and if we monitor the appropriate ions of the CI mass spectrum of the monomethylated derivative, m/e 235 and m/e 237, considerable broadening of peaks occurs as for the underivatized *nor*-nitrogen mustard above. Therefore, we found it much more suitable to monitor the dimethylated derivative (m/e 249 and m/e 251) where the SIM profiles are much sharper and more easily quantitated. The trimethylated derivative can also be used if, as will be illustrated later, there is background interference in the dimethyl derivative profile.

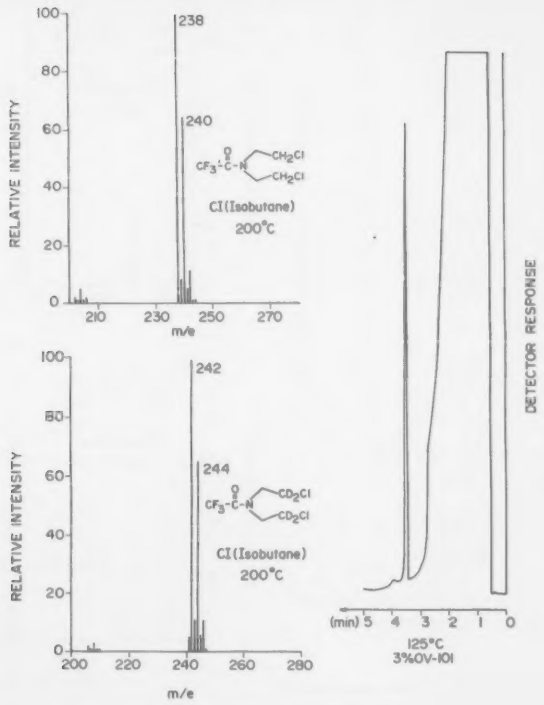


FIGURE 2.—Gas chromatogram and isobutane CI mass spectra of *nor*-nitrogen mustard and d_4-*nor*-nitrogen mustard as their trifluoroacetyl derivatives.

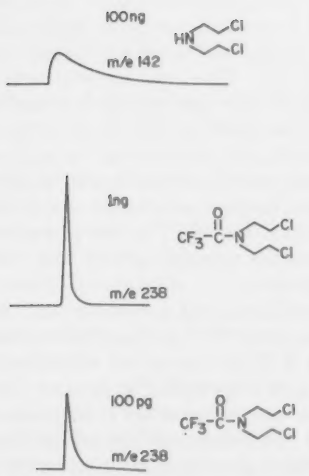


FIGURE 3.—Reduction of tailing with consequent increased detection sensitivity for derivatized (TFA) *nor*-nitrogen mustard relative to the underivatized compound.

Figure 6 shows the SIM profile of standard dimethylphosphoramide mustard using m/e 263 $(M + H)^+$, m/e 265, m/e 267, and m/e 269 where the appropriate peak ratios of 9:6:1 reflect natural chlorine isotope abundances. This ratio provides a useful indication that the correct ions are being monitored and measured, although the gas chromatographic retention time coupled with the specificity of the ions being monitored is really sufficient.

In figure 7 the profiles of ions formed from the derivatized d_4-*nor*-nitrogen mustard are shown to examine the contribution made to those ions (m/e 238 and m/e 240) which will be used to quantitate the undeuterated (unknown) material. The gain on the m/e 238 and m/e 240 ion profiles is 100 times that on the m/e 242 and m/e 244 ion profiles. It is noted that there is a contribution to the m/e 238 and m/e 240 ions but it is extremely small (m/e 238 is $< 0.1\%$ of the abundance of m/e 242, for example) and will generally be negligible if the ratio of unknown to standard is maintained close to 1:1.

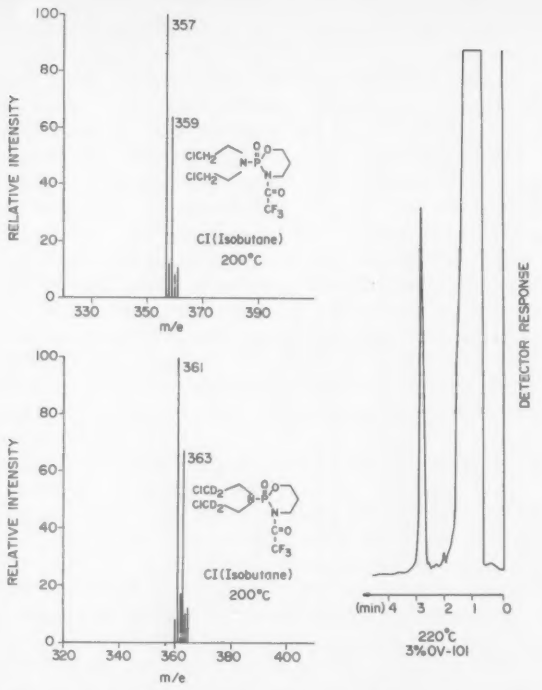


FIGURE 4.—Gas chromatogram and isobutane CI mass spectra of CP and d_4-CP as their trifluoroacetyl derivatives.

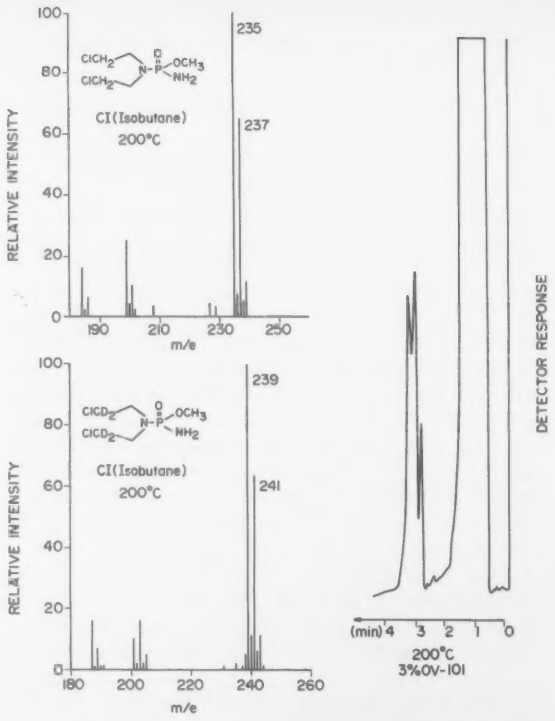


FIGURE 5.—Gas chromatogram of phosphoramide mustard after derivatization with diazomethane and the isobutane CI mass spectra of the monomethylated derivative and d_4-monomethylated derivative.

Since all the standards used here are made from the same building block, this contribution will be the same for all the compounds of interest. Conversely, there will be a contribution to the $(M+H)^+$ ions of deuterated standards when comparable levels of unknown to standard are being measured (fig 6). However, when this is the case the chlorine isotope ions at $(M + 2 + H)^+$ of the deuterated standards are used for quantitation since there is no overlap here from the undeuterated unknown (fig 6).

It is generally advisable to calibrate the methodology and instrumentation in this type of analysis. That is, for each drug or metabolite extracted with an appropriate amount of standard for the range of levels of unknown considered, several determinations are made for each point of a calibration curve. A typical calibration curve is shown in figure 8 for CP extracted from urine doped with deuterated standard. Each point is the mean of five determinations and the range of values for the lowest point is $\sim \pm 10\%$ of the mean. Values referred to below as uncorrected are given without reference to a calibration experiment although any correction is usually small.

CP and *nor*-nitrogen mustard are simultaneously extracted from basic urine or basic plasma (to pH 8.5 with NaOH) and the dried extract is derivatized with TFA (9). That no *nor*-nitrogen mustard is formed from CP during derivatization is easily tested by derivatizing CP along with d_4-*nor*-nitrogen mustard and monitoring for any undeuterated derivatized *nor*-nitrogen mustard. None is produced. This method will be generally useful to test for decomposition of CP and its metabolites to other metabolites in extraction and derivatization procedures (5).

Figure 9 illustrates a typical SIM profile from a patient sample. This is the SIM recording of CP-TFA from a 0–4-hour urine collection of a patient who received a 15-mg/kg iv dose of CP. The profile represents an uncorrected urine concentration of 10 μg/ml. The methods described here routinely produce clear profiles with little or no background interference at the retention times of interest for CP and *nor*-nitrogen mustard extracted from urine or plasma.

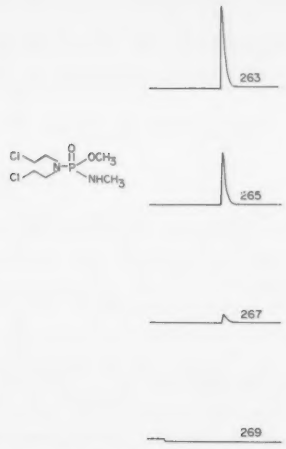


FIGURE 6.—SIM of dimethylphosphoramide mustard ions.

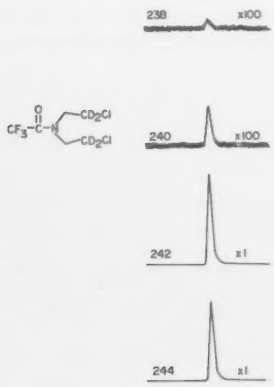


FIGURE 7.—SIM of ions of d_4-*nor*-nitrogen mustard as the trifluoroacetyl derivative.

Table 1 shows the results of a typical experiment to determine the time course of *nor*-nitrogen mustard levels in urine of a patient after a 7.5-mg/kg iv injection of CP. The values are uncorrected.

Phosphoramide mustard is extracted from blood and urine by the use of an XAD-2 column[5] and derivatized with ethereal diazomethane as previously described (5). Figure 10 shows the SIM profiles of the dimethyl and trimethyl derivatives of phosphoramide mustard from a patient urine sample. This illustrates the utility of having more than one derivative since if difficulty is experienced in

[5]Since this paper was prepared, we have found that XAD-2 packing is now made by a different manufacturing process and is not now suitable for this extraction. XAD-4 packing should be used.

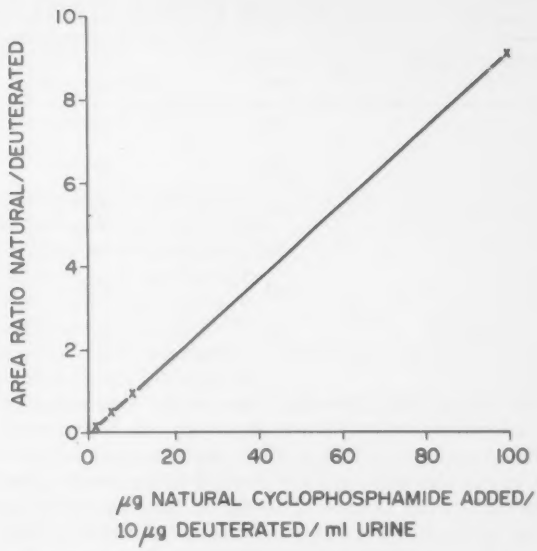


FIGURE 8.—Calibration curve of CP (1-100 μg/ml) extracted from urine doped with 10 μg/ml of d_4-CP.

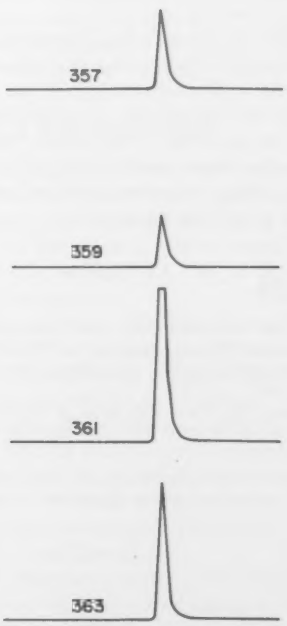


FIGURE 9.—SIM of ions of CP and d_4-CP standard extracted from a patient urine sample.

TABLE 1.—Levels of *nor*-nitrogen mustard in human urine over a 12-hr period after a single iv injection of CP (0.525 g; 7.5 mg/kg)

Collection period (hrs)	Volume (ml)	μg excreted (μg/ml)
0-2	232	348 (1.5)
2-4	253	885 (3.5)
4-6	139	417 (3)
6-8	248	347 (1.4)
8-10	521	1302 (2.5)
10-12	148	326 (2.2)

measuring the dimethyl derivative because of background interference (as in the m/e 251 trace, for example) and tailing, then the trimethyl derivative can be conveniently used. In this particular patient urine sample (0-4-hour collection after 15 mg/kg iv of CP) the appropriate uncorrected levels of CP, *nor*-nitrogen mustard, and phosphoramide mustard were 10, 30, and 15 μg/ml.

Lastly, an example of a recent determination of phosphoramide mustard in a patient plasma extract collected 75 minutes after a 1-hour infusion of 50 mg/kg of CP revealed an uncorrected concentration of 22 μg/ml of phosphoramide mustard from 7.8 ml of plasma.

These levels of *nor*-nitrogen mustard and phosphoramide mustard from these preliminary studies are reasonably high and point to some interesting and hopefully useful studies in quantitating these metabolites in patients. The direct extension of quantitation by these methods to 4-hydroxycyclophosphamide, 4-ketocyclophosphamide, and carboxyphosphamide should be possible.

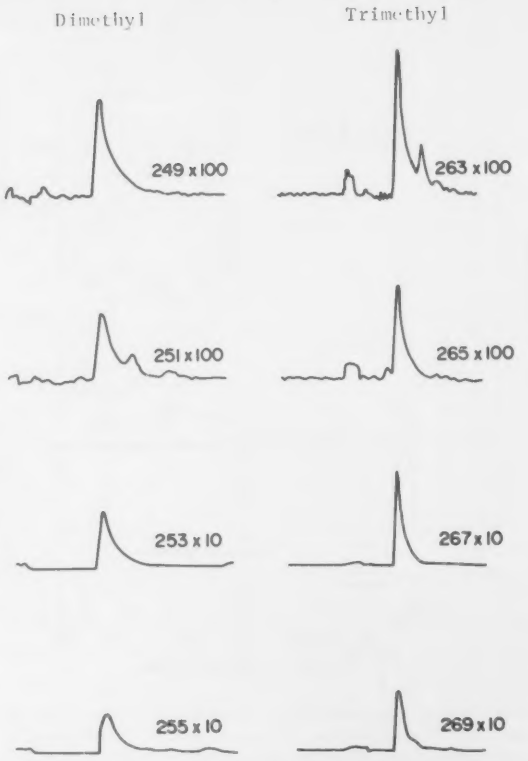


FIGURE 10.—SIM of ions of dimethylated and trimethylated phosphoramide mustard with their corresponding d_4 internal standards extracted from a patient urine sample.

REFERENCES

1. FENSELAU C. Gas chromatography mass spectrometry: a report on the state of the art. Appl Spectry 28:305-318, 1974.
2. MUNSON B. Chemical ionization mass spectrometry. Anal Chem 43:28A-43A, 1971.
3. BEGGS D, VESTAL ML, FALES HM, ET AL. A chemical ionization mass spectrometer source. Rev Sci Instrum 42:1578-1584, 1971.
4. COLVIN M, PADGETT CA, and FENSELAU C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
5. FENSELAU C, KAN MN, BILLETS S, ET AL. Identification of phosphorodiamidic acid mustard as a human metabolite of cyclophosphamide. Cancer Res 35:1453-1457, 1975.
6. FRIEDMAN OM, and SELIGMAN AM. Preparation of *N*-phosphorylated derivatives of bis-β-chloroethylamine. J Am Chem Soc 76:655-658, 1954.
7. FRIEDMAN OM, BOGER E, GRUBLIAUSKAS V, ET AL. Synthesis of *N*-phosphorylated derivatives of nitrogen mustards with latent cytotoxicity. J Med Chem 6:50-59, 1963.
8. JARMAN M, GILBY ED, FOSTER AB, ET AL. The quantitation of cyclophosphamide in human blood and urine by mass spectrometry-stable isotope dilution. Clin Chim Acta 58:61-69, 1975.
9. PANTAROTTO C, BOSSI A, BELVEDERE G, ET AL. Quantitative GLC determination of cyclophosphamide and isophosphamide in biological specimens. J Pharm Sci 63:1554-1558, 1974.

Sparing Effect of Cyclophosphamide (NSC-26271) Pretreatment on Animals Lethally Treated With γ-Irradiation[1]

J. L. Millar* and B. N. Hudspith[2]

SUMMARY

Cyclophosphamide (CP) can protect mice from γ-irradiation-induced lethality, the timing of the dose of CP in relation to the irradiation being the critical factor. The most sparing effect is achieved when CP is injected 3 days before irradiation. The protection is mediated by enhanced hemopoietic recovery seen in animals given the most favorable combination of CP and γ-irradiation. This rapid recovery cannot be explained by the reduced sensitivity of hemopoietic stem cells after CP treatment to subsequent irradiation although survival of a few radioresistant stem cells cannot be ruled out. Stimulus to repopulate by nonspecific depletion of the bone marrow is not the explanation either, since CP itself appears to play an important role in this effect. The role of the spleen is not critical in this phenomenon and attempts have failed to demonstrate the presence of a humoral factor which can restore bone marrow depleted by γ-irradiation in the serum of animals treated with CP. CP given 3 days before irradiation appears to result in a purely additive effect against malignant tissue in mice, providing a useful differential kill on tumor tissue at the same time that it provides the maximum protection of normal tissue.

[Cancer Treat Rep 60:409-414, 1976]

Reduced lethality has been observed in mice receiving a combined dose of cyclophosphamide (CP) and busulfan (1), where the busulfan dose itself (40 mg/kg) would have been lethal. In the case of busulfan-induced death, rescue may be mediated, at least in part, by the CP-induced elaboration of a humoral factor which enhances hemopoietic recovery (1).

Bozduganov (2) has shown that CP can protect mice treated with a lethal dose of irradiation. In addition, it has been shown that hemopoietic stem cells can regenerate more rapidly in animals pretreated with CP than in control animals (3) although there has been no evidence that a humoral factor was involved.

We will present evidence that CP pretreatment protects mice from γ-irradiation-induced lethality and that the timing of the drug in relation to the dose of radiation is critical for increased survival. The protection afforded by CP will be discussed in terms of enhanced recovery of the hemopoietic system and our attempts to elucidate the mechanism of this phenomenon will be reported.

MATERIALS AND METHODS

Animals.—Male or female 8-10-week-old CBA and C57BL mice were used in all experiments. Female mice, weighing between 18 and 24 g, were used as recipients in the spleen colony experiments. Male mice, weighing between 22 and 27 g, were used in all other experiments.

Cytotoxic agents.—CP was dissolved in water and injected as a single ip injection. Irradiation was accomplished using a cobalt 60 (^{60}Co) gamma source at the rate of 60 rads/minute.

CFU assay.—The colony forming units (CFU) content of femurs was assayed using the method of Till and McCulloch (4). Recipients were given 850 rads of ^{60}Co γ-irradiation 4 hours before bone marrow transplantation. Generally, eight mice were used in each group.

Blood leukocyte counts.—The blood leukocyte concentration was measured using a model F Coulter Counter and differentials were performed on ethanol-fixed Giemsa-stained blood films made at

[1]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate. Supplied by W. B. Pharmaceuticals Ltd, Blacknell, Berkshire, England.

[2]Biophysics Division, Institute of Cancer Research, Sutton, England.

*Reprint requests to: Dr. J. L. Millar, Biophysics Division, Institute of Cancer Research, Sutton, Surrey SM2 5PX, England.

the time of sampling. One hundred cells/slide were analyzed.

Iron 59 (^{59}Fe) uptake.—The uptake of ^{59}Fe into newly formed red blood cells (rbc) was used as a measure of erythropoiesis. One microcurie of ^{59}Fe was injected ip into each mouse 2 days before sampling the peripheral blood for activity. Counts were measured on an Intertechnique gamma counter.

Serum.—The collection and treatment of test serum were done as previously described (1).

RESULTS

For CBA male mice exposed to ^{60}Co γ-irradiation, the dose that kills 50% of the animals within 30 days (LD50 [30]) is 925 rads; C57BL male mice are slightly more sensitive (unpublished observation). A dose of 1000 rads will kill all animals of both strains in about 9-12 days (tables 1 and 2). A single dose of 200 mg/kg of CP was given to mice at various times before or after 1000 rads of whole-body irradiation (WBR) (fig 1). In both strains it became apparent that the mice given CP 3 days before irradiation were spared to a considerable extent. The effect was slightly more pronounced in C57BL mice than in CBA mice.

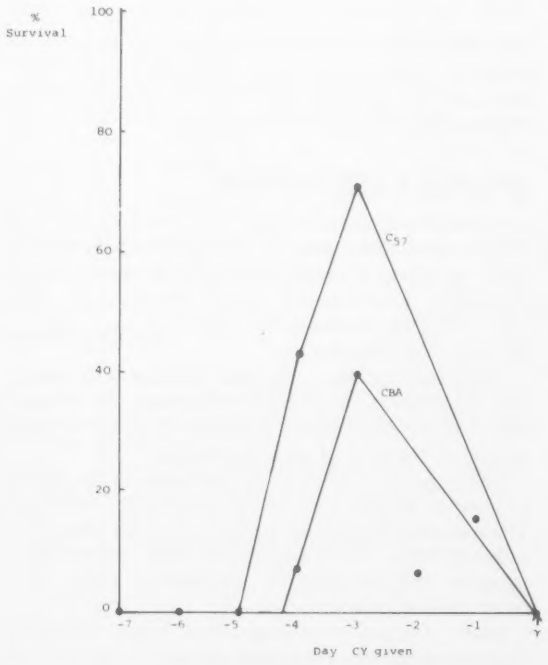


FIGURE 1.—Mouse survival after 200 mg/kg of CP and 1000 rads of WBR. CY = CP.

Effect of CP Treatment Given 3 Days Before γ-Irradiation on the Blood Leukocyte Count and Rbc Production

A reduced dose of 900 rads was used in this study to allow for sufficient survival in the groups that were given irradiation alone. Figure 2 shows that in animals given the optimum combination of CP and irradiation there is a rapid recovery in the hemopoietic system. The recovery of the white blood cell (wbc) count precedes the recovery of the rbc production by about 2-3 days. In contrast, in animals given irradiation alone, there is a decline in the wbc count to a minimum on Day 9 when animals given a higher dose of 1000 rads usually die (tables 1 and 2). Figure 3 shows the time course of the peripheral blood granulocyte count after treatment with the optimum combination of CP and irradiation. After a marked initial depression there is a steady recovery to normal in about 16 days. In animals treated with 900 rads only the neutropenia lasts from Day 4 to Day 14. Animals receiving 200 mg/kg of CP alone undergo a period of intense granulocytosis between Days 5 and 7 after receiving the drug. During this time, the peripheral granulocyte count is almost double that of control animals, but by Day 15 the count has returned to normal.

Radiosensitivity of CFU After CP Treatment

Groups of animals were treated with 200 mg/kg of CP and 3 days later a dose-survival estimate was performed on their femoral CFU using γ-irradiation to see if the CP treatment had altered the sensitivity of these cells to irradiation. In figure 4 the survival of CP-treated CFU has been normalized using the group that received CP without irradiation. Within the experimental error and range investigated, there is no great change in the sensitivity of cells treated with CP as evidenced by the similar slopes of each survival. In absolute terms, of course, the animals pretreated with CP have considerably fewer CFU than untreated controls at each dose level, as CP itself depresses the CFU to about 14% of the normal level.

Irradiation as a Pretreatment to Irradiation and CP as a Pretreatment to CP

A dose of 200 rads was given to groups of mice at various times before and after 1000 rads of WBR; the results are shown in table 1. Survival was independent of the timing of irradiation and there was no increase in the lifespan of any group of animals, suggesting that simply depleting the

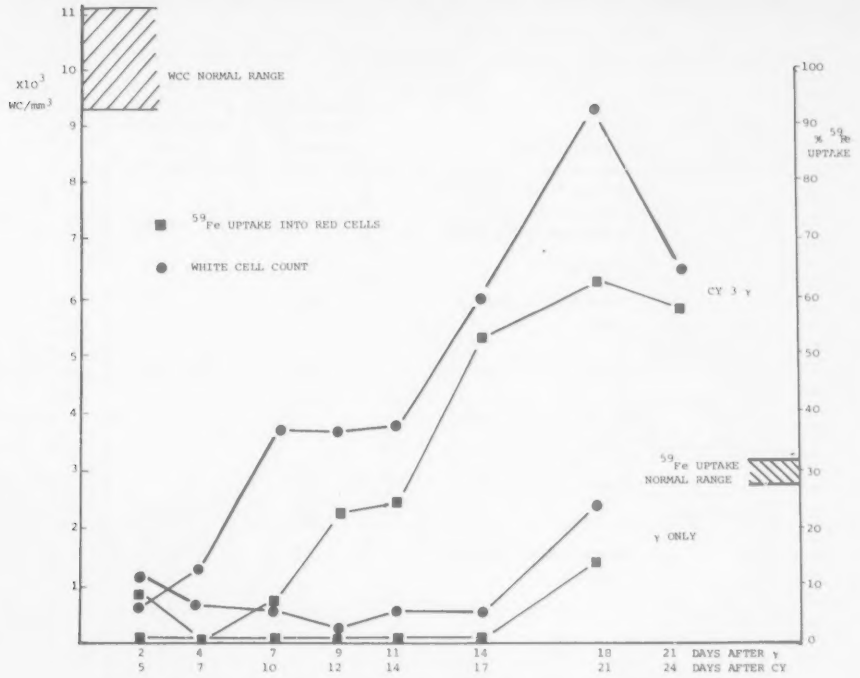


FIGURE 2.—Peripheral blood leukocyte concentration and ^{59}Fe uptake into rbc in mice given 200 mg/kg of CP 3 days before 900 rads of WBR. Cy 3γ indicates that CP was administered 3 days before irradiation.

TABLE 1.—Effect of irradiation used as a pretreatment to irradiation and CP as a pretreatment to CP

Time of "protective" treatment in relation to 2nd treatment	21-day survival when 200 rads was given with 1000 rads of irradiation*	Average day of death of non-survivors (± SE)	21-day survival when 200 mg/kg of CP was given with 200 mg/kg of CP*	Average day of death of non-survivors (± SE)
4 days before	0/10	8.6 ± 0.3	8/14	10.0 ± 3.5
3 days before	0/10	9.7 ± 0.4	12/14	5.5 ± 0.5
2 days before	0/10	10.7 ± 1.0	11/14	13.0 ± 3.6
1 day before	0/10	9.3 ± 0.9	2/14	11.8 ± 1.5
Same day	0/10	9.5 ± 0.6	1/14	11.2 ± 1.5
1 day after	0/10	10.7 ± 0.7	—	—
3 days after	0/10	9.8 ± 0.5	—	—
1000 rads γ-irradiation alone	0/18	11.6 ± 0.2	—	—

*No. of mice surviving/total No. of mice treated.

TABLE 2.—Effect of serum from CP-treated mice on survival of lethally irradiated animals

Experiment No.	Schedule and dose	No. of 30-day survivors/total No. of mice treated	Average day of death of nonsurvivors (± SE)
1	1000 rads WBR followed by 3 daily ½-ml injections of dialyzed serum from:		
	Controls	0/13	9.7 ± 0.6
	CP-pretreated (2 days) mice	0/13	9.5 ± 0.5
	γ-Irradiation-alone mice	0/12	9.2 ± 0.6
2	1000 rads WBR preceded by (4 days before) 3 daily ½-ml injections of dialyzed serum from:		
	Controls	0/8	12.1 ± 0.4
	CP-pretreated (2 days) mice	0/8	14.3 ± 0.7
	γ-Irradiation-alone mice	0/8	11.4 ± 0.3
3	950 rads WBR preceded by (3 days before) 5 daily ½-ml injections of dialyzed serum from:		
	Controls	0/8	11.6 ± 1.0
	CP-pretreated (3 days) mice	0/7	12.3 ± 1.8
	γ-Irradiation-alone mice	1/8	10.3 ± 0.6

bone marrow to the same extent as 200 mg/kg CP does not allow for the protection phenomenon presented in figure 1. Conversely, if CP is used as a pretreatment to CP, considerable sparing is seen if the first dose is administered 3 days before the second dose. Both doses were 200 mg/kg; the combined dose of 400 mg/kg proved fatal when administered as a single injection (table 1).

Effect of Splenectomy on the Sparing Effect of CP Pretreatment

The role of the spleen in the effectiveness of CP pretreatment of animals receiving a lethal dose of irradiation was investigated. Animals were splenectomized from 2 weeks to 5 months before the beginning of the experiment. Table 3 indicates that if the spleen plays a role in the phenomenon of CP protection then it is not a vital one because splenectomized animals given CP as a pretreatment have a better survival rate than splenectomized controls.

Experiments Involving Serum From CP-Treated Animals

There is no evidence so far that serum taken from animals treated with CP 2 or 3 days previously can restore hemopoiesis in animals depleted by γ-irradiation (table 2). Test sera were injected at various times before or after the irradiation as indicated in the table. There was no survival in the treated groups even at the reduced dose of 950 rads (experiment 3) and no significant increase in lifespan.

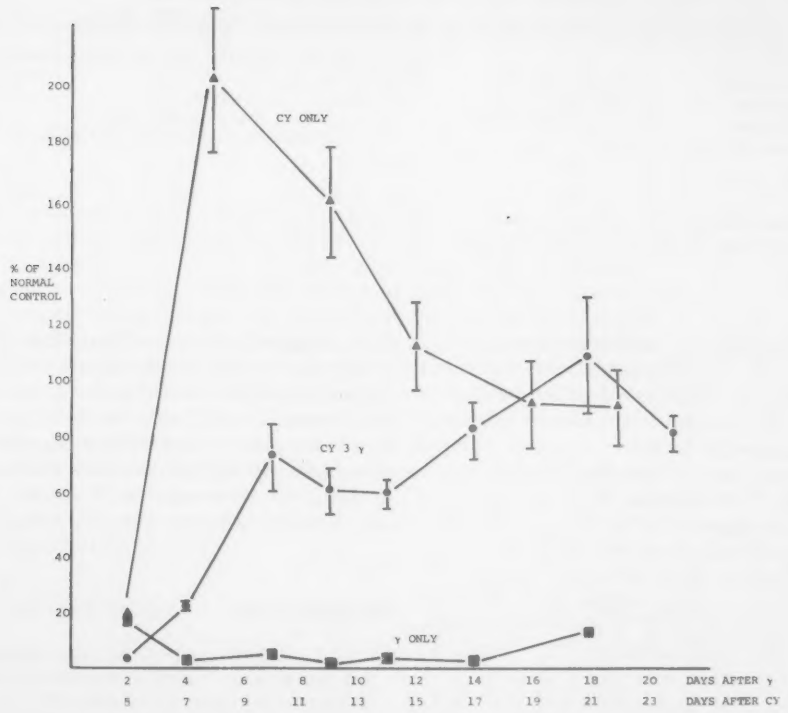


FIGURE 3.—Peripheral blood granulocyte concentration after 200 mg/kg of CP and/or 900 rads of WBR. Cy 3γ indicates that CP was administered 3 days before irradiation.

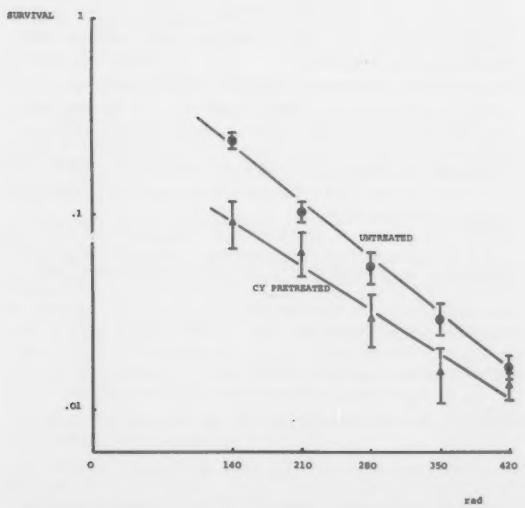


FIGURE 4.—Sensitivity of femoral CFU to γ-irradiation in animals pretreated with CP. CY = CP.

DISCUSSION

CP pretreatment spares animals receiving an otherwise lethal dose of busulfan (1). We have shown that a similar sparing effect is achieved if CP is given at an appropriate time before a dose of γ-irradiation in excess of the LD100 (30). This protection comes about through enhanced recovery of hemopoiesis in CP-treated animals. However, CFU data indicate that CP treatment does not make the majority of bone marrow stem cells resistant to subsequent irradiation and therefore the protective effect is probably not due to greater stem-cell survival in CP-treated animals. Valeriote et al have shown (5) that CFU and hemopoietic recovery have been faster in CP-treated animals than in irradiated animals where the initial depression of CFU has been the same. It could be postulated that a CP-induced factor enhances hemopoiesis in CP- but not in γ-irradiation-depleted bone marrow, although such a factor could not be demonstrated in our system. There has also been evidence that recovery of transplanted stem cells

TABLE 3.—Role of the spleen on the sparing effect of CP pretreatment

Time before treatment that CBA male mice were splenectomized	30-day survival after CP (200 mg/kg) 3 days before 1000 rads WBR*	Average day of death of non-survivors (± SE)	30-day survival after 1000 rads WBR alone*	Average day of death of non-survivors (± SE)
5 mos	2/5	12.7 ± 0.7	0/5	13.6 ± 0.7
2 wks	3/10	25.4 ± 0.8	0/9	12.9 ± 0.5

*No. of mice surviving/total No. of mice treated.

takes place more rapidly in recipient animals pretreated with CP (3). Furthermore, bone marrow cells implanted ip in diffusion chambers exhibit enhanced growth if recipients are CP pretreated (6), suggesting a humoral factor.

In our experience, perturbing the bone marrow with 200 rads of γ-irradiation does not protect animals from a subsequent lethal dose of irradiation. In contrast, animals given 400 mg/kg of CP in two 200-mg/kg doses 3 days apart are protected from what would be a lethal single dose. In this instance the second dose of CP is given at a time when the CFU are most sensitive (7). Thus, it seems likely that the rate of recovery of CFU is probably more important for an animal's survival than the absolute level of initial depression.

Preliminary investigations have been carried out in male C57BL mice bearing the Lewis lung tumor to test the combination of CP and γ-irradiation in tumor-bearing animals. The protective effect of CP 3 days before 1000 rads of γ-irradiation was again observed (unpublished observation). The result suggests that any hematologic changes which take place in tumor-bearing animals do not alter the basic mechanism by which CP pretreatment enhances hemopoietic recovery. The efficacy of CP and γ-irradiation against tumor tissue does not change, remaining additive, within the time period in which CP protects the bone marrow.[3] This suggests that a differential effect against tumor tissue may be obtained if CP and γ-irradiation are timed to minimize damage against normal tissue. It is feasible that larger doses of γ-irradiation may be administered under these conditions than would otherwise be tolerated, thus increasing the effect against the tumor. Experiments are in progress to test this hypothesis.

[3]Steel GG. Personal communication.

REFERENCES

1. MILLAR JL, HUDSPITH BN, and BLACKETT NM. Reduced lethality in mice receiving a combined dose of cyclophosphamide and busulphan. Br J Cancer 32:193-198, 1975.
2. BOZDUGANOV A. Radioprotection and cyclophosphamide. *In* Biological Aspects of Radiation Protection (Sugahara T, and Hug O, eds). Tokyo, Shoin, 1971.
3. FRIED W, HUSSEINI S, GREGORY S, ET AL. Effect of cyclophosphamide on the haematopoietic microenvironmental factors which influence hematopoietic stem cell proliferation. Cell Tissue Kinet 6:155-163, 1973.
4. TILL JE, and MCCULLOCH EA. A direct measurement of the radiation sensitivity of natural mouse bone marrow cells. Radiat Res 14:213-222, 1961.
5. VALERIOTE FA, COLLINS DC, and BRUCE WR. Hematological recovery in the mouse following single doses of gamma radiation and cyclophosphamide. Radiat Res 33:501-511, 1968.
6. STOHLMAN F, QUESENBERRY P, NISKANIN E, ET AL. Control of granulopoiesis. *In* Hemopoietic Stem Cells (Wolstenholm GEW, and O'Connor M, eds). CIBA Foundation Symposium 13 (New Series), Amsterdam, 1973.
7. DEWYS WD, GOLDIN A, and MANTEL N. Hematopoietic recovery after large doses of cyclophosphamide: correlation of proliferative state with sensitivity. Cancer Res 30:1692-1697, 1970.

Identification and Pharmacokinetics of Cyclophosphamide (NSC-26271) Metabolites In Vivo[1,2]

G. Voelcker,* T. Wagner, and H.-J. Hohorst[3,4]

SUMMARY

The existence of the cyclophosphamide (CP) metabolites, 4-hydroxycyclophosphamide and aldophosphamide, in vivo after application of CP to mice and rats has been demonstrated. The metabolite pattern in the serum and urine of rats at different times after CP application was determined. For this purpose, a thin-layer chromatographic procedure was developed which allows separation of the metabolites without decomposition. Because considerable amounts of radioactivity are bound to serum proteins after ^{3}H-CP application, protein binding studies with CP and CP metabolites were carried out. It was found that 4-hydroxycyclophosphamide is bound to thiol groups of bovine serum albumin yielding a rather stable product which could be isolated by column chromatography on Sephadex.

[Cancer Treat Rep 60:415-422, 1976]

MATERIALS AND METHODS

^{3}H-cyclophosphamide (CP) was purified by thin-layer chromatography (TLC) on 1,3-propandiol-impregnated cellulose plates with ethyl acetate as a mobile phase as has been reported (1). ^{3}H side-chain labeled 4-hydroxycyclophosphamide (4-hydroxy-CP) (specific activity, 0.1 microcurie/μmol) was prepared by reduction of ^{3}H side-chain labeled 4-hydroperoxycyclophosphamide with triphenyl phosphine in CH_2Cl_2 as previously reported (1,2).

TLC.—The determination of CP, 4-hydroxy-CP, aldophosphamide, and carboxyphosphamide by TLC on propandiol-impregnated cellulose plates was carried out as has been described (1). *Nor*-nitrogen mustard and phosphoramide mustard were analyzed by TLC on pre-coated cellulose plates (Merck 5716) with butanol:glacial acetic acid: water (80:11:20) as a mobile phase. 4-Ketocyclophosphamide (4-keto-CP) and 4-(*S*-benzyl)-mercaptocyclophosphamide (4-[*S*-benzyl]-mercapto-CP) were detected by TLC on pre-coated silica gel plates (Merck 5721) with ethyl acetate as a mobile phase.

Thin-layer electrophoresis[5].—Thin-layer electrophoresis was carried out at 2°C on pre-coated cellulose plates which were impregnated with a buffer solution of pyridine:glacial acetic acid:water (10:1:89, pH 6.5); the field strength was 27 volts/cm.

Evaluation of developed chromatograms and electrophoresis plates.—For detection of alkylating substances, thin-layer chromatograms and electrophoresis plates were sprayed with a 2.5% solution of 4-(*p*-nitrobenzyl)pyridine (NBP) in an acetone-acetate buffer (1:1), heated for 10 minutes at 150°C, and then sprayed with 0.25 N NaOH. Radioactive bands were located either with a Berthold radiochromatogram scanner or by liquid scintillation counting of 0.1-inch bands of the absorbent scraped off at right angles to the direction of development.

Isolation of carboxyphosphamide.—After the addition of several volumes of ethanol to the urine of a patient treated with ^{3}H side-chain labeled CP,

[1]Supported by Bundesministerium für Forschung und Technologie.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate. ^{3}H side-chain labeledCP (180 microcuries/μmol) was a gift of Prof. Schaumlöffel, Klinik und Polyklinik für Nuclearmedizin, Marburg, Germany; phosphoramide mustard, *nor*-nitrogen mustard, non-labeled CP, and 4-ketocyclophosphamide were gifts of Prof. Brock, Asta-Werke AG, Brackwede, Germany.

[3]Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany.

[4]We wish to thank Miss Anke Wilkoewitz for her excellent assistance.

*Reprint requests to: Dr. G. Voelcker, Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, Theodor-Stern-Kai 7, 6000 Frankfurt, 70 West Germany.

[5]Pherograph Original Frankfurt Type Mini 65 made by Hormuth and Vetter, Wiesbaden, Germany.

the urine-ethanol mixture was evaporated under reduced pressure. After removal of the insoluble material by centrifugation, the supernatant was separated on 1,3-propandiol-impregnated cellulose plates (1). Carboxyphosphamide was located on the developed chromatogram by means of a standard applied to an indicator strip and visualized by NBP spray. The carboxyphosphamide-containing cellulose was removed and eluted with CH_2Cl_2.

Determination of radioactivity.—Radioactivity was measured by liquid scintillation counting in a Philipps scintillation spectrometer (PW 45/0/01). A scintillation system containing 2 g of 2-(4-*t*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (butyl-PBD) in 1 liter of toluene-methylglycol mixture (6:4) was used.

Colorimetric estimation of alkylating substances.—The concentration of alkylating substances was determined after acid hydrolysis in 1 N HCl at 100°C for 10 minutes as described by Friedman and Boger (3). The total alkylating and potentially alkylating material has been designated as the alkylating capacity. The rate of decrease of the alkylating capacity, as determined by NBP assay, is proportionate to the alkylating activity of the substance studied.

Protein binding studies by equilibrium dialysis.—Protein binding studies were carried out at 4°C in an equilibrium dialyzing system described elsewhere (4). Teflon half cells (1.36 ml) separated by a membrane (Union Carbide G 13) were used; 40 mg/ml of crystallized bovine albumin was dissolved in 0.07 M phosphate buffer (pH 7) containing 0.5% NaCl (ionic strength 0.23). The corresponding teflon half cells of the dialyzing system were filled with buffer and protein solution. After temperature adjustment, 5-20 μl of the substance studied dissolved in buffer was introduced into the half cell containing the protein solution. The teflon cells were rotated at 10 rpm. Preliminary experiments showed that equilibrium was reached after 3 hours at 4°C. After an incubation period of 6 hours the 4-hydroxy-CP concentration of both of the half cells was measured by liquid scintillation counting and the concentrations of bound and free ligand were calculated.

Determination of sulfhydryl groups.—The thiol content of bovine albumin was determined spectrophotometrically by titration with 4,4′-dithiopyridin (5) (Aldrich, Europe, Beerse, Belgium). For calculation, a molecular weight of 66,000 (6) was used for bovine serum albumin; we determined 0.67 mol thiol groups/mol of bovine serum albumin.

Protein determinations.—The concentration of bovine serum albumin solution was determined by measuring the optical density at 280 nm. The protein concentration of serum samples was determined by biuret-reaction (7).

Estimation of CP metabolism.—Rats (SPF, Sprague-Dawley strain, 350-400 g) were injected ip with ^3H side-chain labeled CP. The serum of blood samples (approximately 50 μl) collected from the tail vein was deproteinized by the addition of the same volume of ice-cold ethanol. After centrifugation, 10 μl of the clear supernatant was applied to propandiol-impregnated cellulose plates by means of an applicator (Chromatocharger, according to Firmenich made by Camag, Muttens, Switzerland). In another series of experiments, when the metabolite pattern of serum and urine was determined simultaneously, the rats were laparotomized after pretreatment with urethane (1.47 g/kg) by the sc route; the bladder was punctured and the urine was removed by a polyvinylchloride tube. The urethra was ligated. Blood samples were drawn from the tail vein. ^3H-CP was injected through a catheter inserted into the right jugular vein.

RESULTS

Figure 1 shows the radio-trace of chromatograms of deproteinized serum of a rat and a mouse which were treated with ^3H side-chain labeled CP (for further details, see fig 1). The trace shows peaks with the following R_f values: 0, 0.13, 0.23, 0.35, 0.53, and 0.71. The fraction with the R_f value of 0.53 behaves chromatographically like CP and 4-keto-CP and those with R_f values of 0.35 and 0.23 respond like 4-hydroxy-CP and carboxyphosphamide respectively. The following characteristics prove that the fraction R_f 0.13 is aldophosphamide:

a. existence of an equilibrium between 4-hydroxy-CP and the fraction in question in 0.07 M phosphate buffer (pH 7) at 37°C and 4°C (1);
b. re-chromatography of the eluted fraction reveals both the compound applied and 4-hydroxy-CP (1);
c. when incubated at 37°C (pH 7) equimolar amounts of acrolein are released (1);
d. both 4-hydroxy-CP and the fraction R_f 0.14 react with benzylmercaptan yielding chromatographically identical reaction products, the structure of which was determined by mass spectroscopy as 4-(*S*-benzyl)-mercapto-CP (8);
e. it has been determined that the fraction in question is not one of the 4-hydroxy-CP diastereomeres or 4-ethoxycyclophosphamide (4-ethoxy-CP) or an acetal formed by the reaction of aldophosphamide with 1,3-propandiol because all these products (the identity of

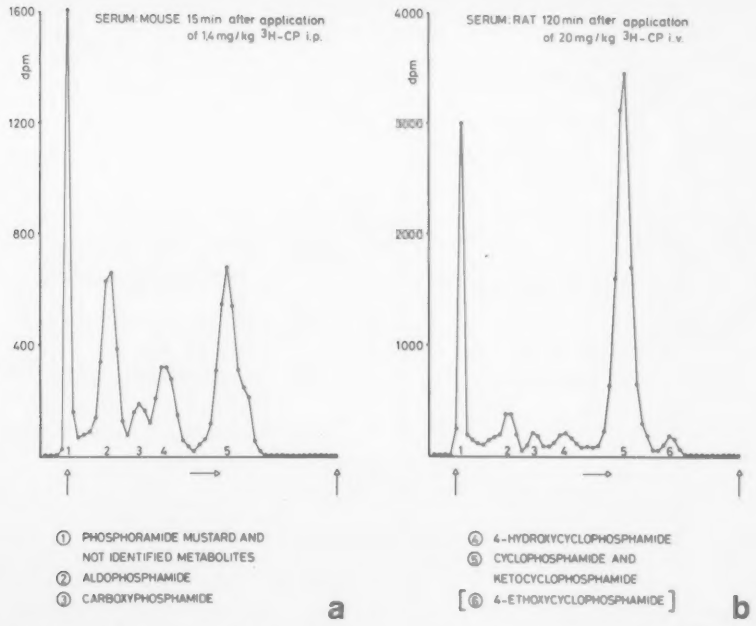


FIGURE 1.—Radio-trace of deproteinized serum after chromatography on 1,3-propandiol-impregnated cellulose plates (metabolite pattern after CP application). (*a*) A mouse was treated ip with 1.4 mg/kg of ^{3}H-CP; 15 min after CP application deproteinized serum was applied to 1,3-propandiol-impregnated cellulose plates and the radioactivity of cellulose bands was determined (*see* Materials and Methods section). (*b*) A rat in urethane-anesthesia (*see* Materials and Methods section) was treated iv with 20 mg/kg of ^{3}H-CP; 120 min after CP application a blood sample was drawn from the tail vein and the serum was deproteinized with ethanol, stored for 5 days at −20°C, and applied to 1,3-propandiol-impregnated cellulose plates.

which was proved by mass spectroscopy [8]) have different R_f values when separated by TLC on 1,3-propandiol-impregnated cellulose sheets with ethyl acetate as a mobile phase. All attempts to identify the fraction R_f 0.14 directly by mass spectroscopy have so far failed due to its rapid decomposition when inserted into the mass spectrometer (8).

The radio-trace of the rat serum shows an additional spot with an R_f value of 0.71. This fraction is 4-ethoxy-CP, the identity of which was proved by mass spectroscopy and which was formed when the deproteinized serum (deproteinization with ethanol) was stored for 5 days at −20°C.

The reactivity of 4-hydroxy-CP and aldophosphamide with mercapto compounds was used as an alternative experimental procedure to prove the existence of these metabolites in mouse serum. The serum was extracted with CH_2Cl_2 and the extract was treated with benzylmercaptan in the presence of trichloroacetic acid. After evaporation under reduced pressure the reaction mixture was separated by TLC on silica gel with ethyl acetate as a mobile phase. Approximately 20% of all the radioactivity was found in a fraction (R_f 0.78) which behaves chromatographically like 4-(*S*-benzyl)-mercapto-CP. Thus, the existence of 4-hydroxy-CP and aldophosphamide in the mouse serum was proved by two independent procedures. In order to identify the compound at the application point of 1,3-propandiol-impregnated cellulose plates the serum of a rat treated ip with ^{3}H-CP was analyzed on cellulose plates with butanol, glacial acetic acid, and water as a mobile phase.

Figure 2 shows a radio-trace of such a chromatogram. The compound with the R_f value of 0.51 is chromatographically identical with phosphoramide mustard. All other fractions except the peak near the solvent front occur after decomposition of phosphoramide mustard in phosphate buffer (pH 7). Analysis of the fraction at the application point of 1,3-propandiol-impregnated cellulose plates by thin-layer electrophoresis at pH 6.5 reveals two fractions: one unknown which behaves electroneutrally and one which migrates to the anode-like phosphoramide mustard. All attempts to detect *nor*-nitrogen mustard failed. Neither by TLC nor by thin-layer electrophoresis could *nor*-

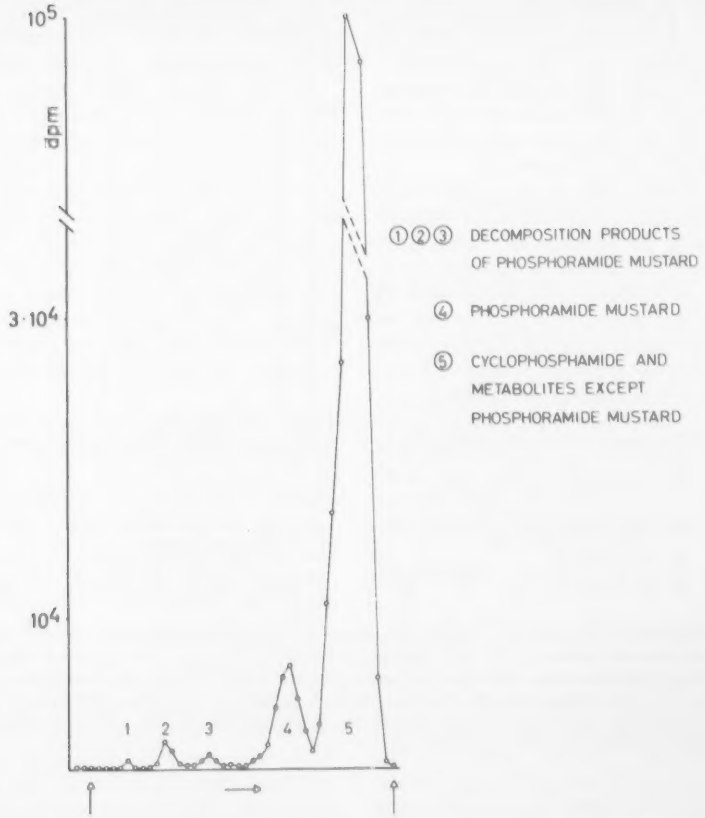


FIGURE 2.—Radio-trace of deproteinized serum after chromatography on cellulose plates with butanol:glacial acetic acid:water (80:11:20) as a mobile phase. Sixty min after ip treatment with 64 mg/kg of ^{3}H-CP the serum of a rat was deproteinized with several volumes of ice-cold ethanol; after removal of insoluble material by centrifugation, the supernatant was evaporated under reduced pressure by cooling with ice. The residue was extracted with ethanol and separated by chromatography on cellulose plates with butanol:glacial acetic acid:water as a mobile phase.

nitrogen mustard be detected as a metabolite of CP. This could also be concluded from kinetic studies at 37°C (pH 7).

Figure 3 shows the decomposition kinetics of *nor*-nitrogen mustard, 4-hydroxy-CP, and phosphoramide mustard when measured by decrease of alkylating and potentially alkylating substances by means of NBP test after acid hydrolysis. The half-lives and rate constants were calculated on the assumption of first-order kinetics. The half-lives calculated from the decomposition kinetics are: *nor*-nitrogen mustard, 380 minutes; 4-hydroxy-CP, 180 minutes; and phosphoramide mustard, 48 minutes. The almost eightfold higher alkylating activity of phosphoramide mustard compared with *nor*-nitrogen mustard demonstrates that the latter cannot be a direct intermediate in the alkylating route under these conditions.

As Brock and Hohorst have reported (9), the chloride-release from *nor*-nitrogen mustard at pH 7 increases with increasing bicarbonate concentrations and is accompanied by a decrease of toxicity of the solution. Arnold and Bekel (10) have demonstrated that this decrease of toxicity is due to formation of 3-(2-chloroethyl)-1,3-oxazolidin-2-one. Since the oxazalidon is not an alkylating agent the oxazalidon formation may be analyzed by means of NBP test. *Nor*-nitrogen mustard was dissolved in serum; after 2 minutes only 50% of the dissolved substance could be determined by means of NBP test and after 15 minutes alkylating material was no longer detectable (*nor*-nitrogen mustard concentration, 0.07 mM).

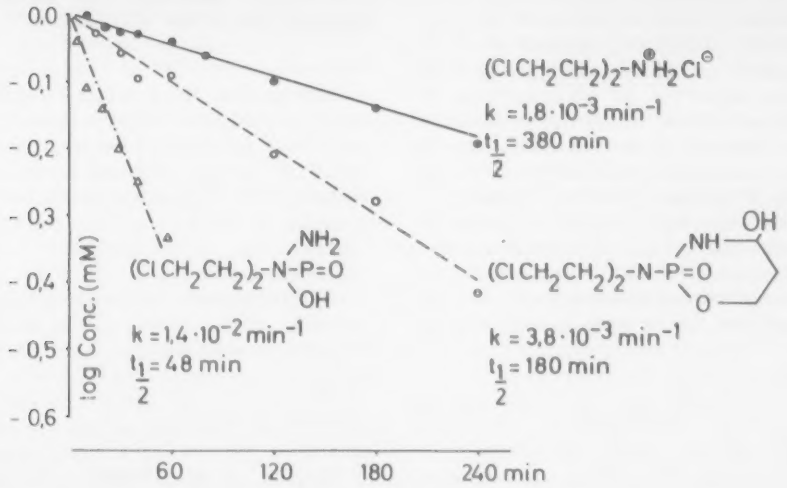


FIGURE 3.—Decomposition kinetics (decrease of alkylator concentration) of *nor*-nitrogen mustard, 4-hydroxy-CP, and phosphoramide mustard in 0.07 M phosphate buffer (pH 7) at 37°C; determination of alkylating capacity by means of NBP test of aliquots.

TABLE 1.—Metabolic pattern in deproteinized rat serum and urine*

Time after ^{3}H-CP treatment (min)	Sum of CP and metabolites (nmol/ml)	Amount (nmol/ml) of CP and CP metabolites				
		CP and 4-keto-CP	4-Hydroxy-CP	Aldophosphamide	Carboxyphosphamide	Other metabolites†
			Serum			
15	46	36	5	1	2	2
30	36	25	5	1	2	3
60	43	30	5	1	3	4
120	43	25	4	5	4	5
180	40	22	3	4	6	5
240	38	20	2	3	6	5
			Urine			
15	337	207	47	63	—	24
30	492	172	30	49	172	69
60	824	141	59	50	422	158
120	1945	78	—	—	1444	430
180	2732	163	—	—	1833	740
240	3634	36	—	—	2593	1019

*Male rat (380 g) was treated iv with 20 mg/kg of ^{3}H-CP (specific radioactivity, 24.5 microcuries/μmol) in urethane anesthesia.
†Phosphoramide mustard and unidentified metabolites.

This experiment indicates that if *nor*-nitrogen mustard was produced in the serum it would be detoxified by the oxazalidon reaction very rapidly which is why it cannot be responsible for cytotoxic activity.

After the identification of CP metabolites in serum in vivo, the metabolic pattern in serum and urine of rats was measured at different times after ^{3}H-CP application. The results are summarized in table 1.

Figure 4 shows the relative distribution of CP and its metabolites 4-hydroxy-CP, aldophosphamide, carboxyphosphamide, and the sum of phosphoramide mustard and unidentified metabolites which cannot be separated on 1,3-propandiol-impregnated cellulose plates. In urine, measurable amounts of the biologically active metabolites 4-hydroxy-CP and aldophosphamide were found only during the first 60 minutes after CP application, whereas in serum the relative concentration of these metabolites remained nearly constant up to 4 hours after CP application. The chromatographic technique did not allow distinction between CP and 4-keto-CP. No attempts were made to determine 4-keto-CP.

Differences Between the Serum and Urine Metabolites of Rat and Man

Until now we have done only one experiment to determine the metabolite pattern in the serum and urine of a patient treated with 30 mg/kg of ^{3}H-labeled CP. In this instance we could not detect 4-hydroxy-CP, aldophosphamide, or carboxyphosphamide in the serum after deproteinization with ethanol but large quantities of carboxyphosphamide were found in the urine. In the patient's urine, the relative concentration of carboxyphosphamide, when referred to the total amount of radioactive materials, increased from 25% to 50%

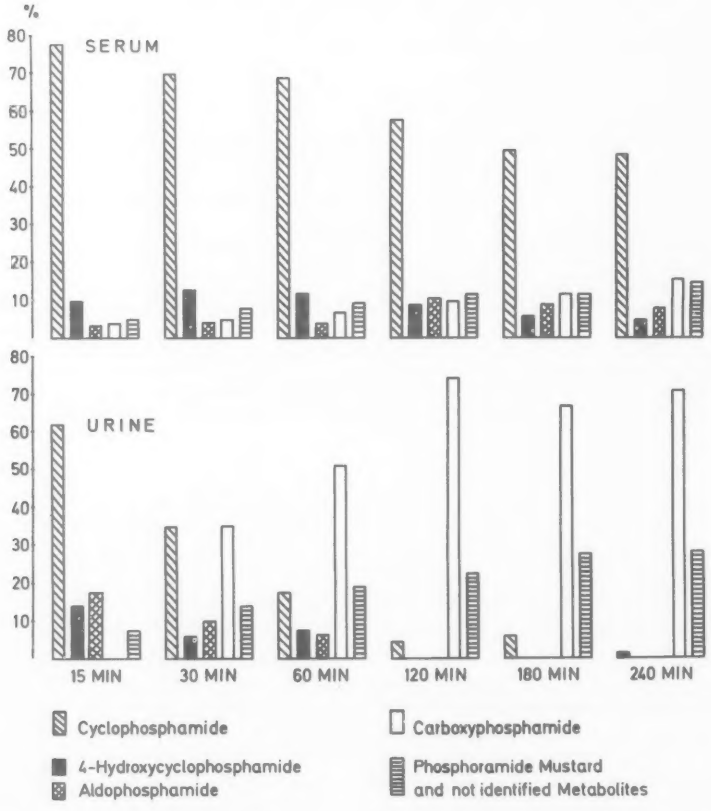


FIGURE 4.—Comparison of the metabolic pattern in the serum and urine of a rat after ip application of 20 mg/kg of ^{3}H-CP. For original concentration of the metabolites *see* table 1.

within 5 hours after CP application and the relative concentration of the nonmetabolized CP in the urine decreased from 58% to 31% during the same time interval. This not only shows that minor activation of CP by liver microsomes might be responsible for the fact that no 4-hydroxy-CP or aldophosphamide could be detected in serum but also that higher detoxification to carboxyphosphamide may have occurred.

Protein Binding Studies With 4-Hydroxy-CP

Equilibrium dialysis of serum in man shows that within 5 hours after ^{3}H-CP treatment 11%-14% of all the radioactivity is bound to serum proteins. Column chromatography on Sephadex G 25 of the 60-minute serum reveals a protein to which 0.15 nmol of radioactive material/mg of protein is bound, that is, 2.6% of all the radioactive material in the serum. Concentration of the protein by ultrafiltration in collodium bags and rechromatography on Sephadex G 25 diminishes the amount of radioactivity bound to protein. In four consecutive separation procedures, the radioactive material decreased from 0.15 to 0.02 nmol/mg of protein. Since CP is not bound to serum albumin (as can be shown by equilibrium dialysis with bovine serum albumin), the bound radioactive materials are CP metabolites.

Binding studies with ^{3}H-labeled carboxyphosphamide (isolated from the patient's urine) by means of equilibrium dialysis show that carboxyphosphamide can also be bound to bovine serum albumin. When the protein/carboxy mixture is separated on Sephadex G 25, no detectable amounts of radioactivity can be found associated with the protein. These findings demonstrate that at least a part of the protein-bound radioactive materials cannot be carboxyphosphamide. Because of the known reactivity of 4-hydroxy-CP with thiol groups which has been described in a subsequent paper (11), we studied the reactivity of 4-hydroxy-CP with bovine serum albumin. Figure 5 shows that 50% of all titratable protein thiol groups are occupied within 10 minutes at 37°C. Obviously, the disappearance of thiol groups of bovine albumin when incubated with 4-hydroxy-CP is not due to alkylation reaction as shown in the following experiment: 4-hydroxy-CP was incubated with bovine serum albumin at 4°C for 6 hours and then separated by column chromatography on Sephadex G

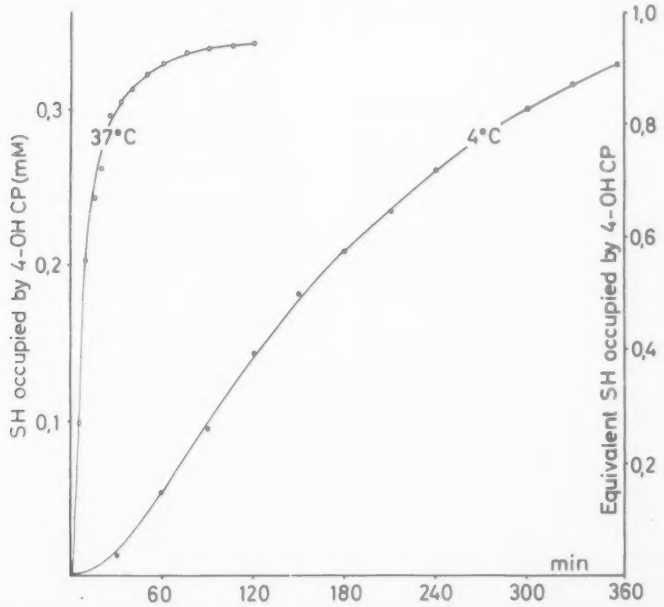


FIGURE 5.—Kinetics of the reaction of titratable thiol groups of bovine albumin with 4-hydroxy-CP (4-OH CP) at 37°C and 4°C. 4-Hydroxy-CP and bovine serum albumin were incubated in 0.07 M phosphate buffer (pH 7); sulfhydryl (SH) determination of aliquots with 4,4'-dithiopyridine (see Materials and Methods section). 4-Hydroxy-CP concentration = 0.7 mM; bovine serum albumin concentration = 0.6 mM corresponding to a thiol concentration of 0.36 mM. In order to determine the spontaneous oxidation rate of thiol groups the thiol concentration of blanks was determined; it was found that under the conditions of the test, 6% of all titratable thiol groups were oxidized spontaneously within 120 min at 37°C and 11% within 6 hr at 4°C.

25. The specific radioactivity of the protein/4-hydroxy-CP reaction product was compared with the specific radioactivity of the original 4-hydroxy-CP. The specific radioactivity of the protein/4-hydroxy-CP reaction product and the specific radioactivity of the original 4-hydroxy-CP were referred to alkylator concentration. The alkylator concentration was measured by means of NBP test. The specific radioactivity of the protein reaction product was determined in three experiments to be 217, 204, and 215 dpm/nmol. The radioactivity of the original 4-hydroxy-CP was 219 dpm/nmol. This indicates that the alkylating capacity of the original 4-hydroxy-CP remains unchanged after protein binding, ie, that no alkylation reactions have occurred.

The molar ratio of 4-hydroxy-CP to protein determined in four experiments was 0.5, 0.43, 0.47, and 0.56 which agrees with the assumption that 4-hydroxy-CP occupies the thiol group of the bovine albumin (the fractional titer of the protein thiol groups was 0.67).

The half-life of the 4-hydroxy-CP/protein reaction product in 0.07 M phosphate buffer (pH 7) at 37°C is 18 hours measured by the decrease of alkylating activity. The corresponding half-life of the original 4-hydroxy-CP is 3 hours. This indicates that the alkylating activity is obviously reduced.

DISCUSSION

This study describes the unequivocal identification of 4-hydroxy-CP, aldophosphamide, and phosphoramide mustard in serum of rats and mice. Contrary to Struck et al (12), *nor*-nitrogen mustard could not be determined in rat and mouse serum. It seems likely that different preparation procedures and TLC techniques may be responsible for that finding. The lack of measurable amounts of 4-hydroxy-CP and aldophosphamide in the patient's serum and urine cannot be interpreted as a result of a qualitatively different metabolic pathway but it shows that there might be a difference between activation and deactivation in laboratory animals and man.

The different metabolic patterns and pharmacokinetics may be the reason for the different chemotherapy responses between laboratory animals and man because 4-hydroxy-CP and aldophosphamide have been shown to bear solely on the oncostatic specificity of CP (13). This, as well as the importance of protein binding of CP metabolites, will be the subject of further investigations.

REFERENCES

1. Voelcker G, Draeger U, Peter G, et al. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
2. Takamizawa A, Matsumoto S, Iwata T, et al. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
3. Friedman OM, and Boger E. Colorimetric estimation of nitrogen mustard in aqueous media. Anal Chem 33:906-910, 1961.
4. Weder HG, Schildknecht J, and Kesselring P. A new equilibrium dialysing system. Am Lab 10:15, 1971.
5. Grassetti DR, and Murray JF, Jr. Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiopyridine. Arch Biochem Biophys 119:41-49, 1967.
6. Carlsson J, and Svenson A. Preparation of bovine mercaptalbumin by means of covalent chromatography. FEBS Lett 42:183-185, 1967.
7. Rafael J, Klaas D, and Hohorst H-J. Mitochondrien aus braunem Fettgewebe: Enzyme und Atmungskettenphosphorylierung während der prä- und postnatalen Entwicklung des intrascapularen Fettkörpers des Meerschweinchens. Hoppe Seylers Z Physiol Chem 349:1711-1724, 1968.
8. Przybylski M, Ringsdorf H, Voelcker G, et al. Characterization of cyclophosphamide (NSC-26271) metabolites and related derivatives by field desorption and electron-impact mass spectrometry. Cancer Treat Rep 60:509-516, 1976.
9. Brock N, and Hohorst H-J. Reactions of bis-(chloroethyl)amine in vitro and in vivo. Arzneim Forsch 11:164-169, 1961.
10. Arnold H, and Bekel H. Die Oxazolidon-Reaktion von 2-Chloräthyl-substituierten sekundären Aminen. Arzneim Forsch 14:750-752, 1964.
11. Draeger U, Peter G, and Hohorst H-J. Deactivation of cyclophosphamide (NSC-26271) metabolites by sulfhydryl compounds. Cancer Treat Rep 60:355-359, 1976.
12. Struck RF, Kirk MC, Witt MH, et al. Isolation and mass spectral identification of blood metabolites of cyclophosphamide: evidence for phosphoramide mustard as the biologically active metabolite. Biomed Mass Spectrom 2:46-52, 1975.
13. Hohorst H-J, Draeger U, Peter G, et al. Problem of oncostatic specificity of cyclophosphamide (NSC-26271): studies on reactions that control the alkylating and cytotoxic activity. Cancer Treat Rep 60:309-315, 1976.

Permeation of Cyclophosphamide (NSC-26271) Metabolites Into Tumor Cells[1,2]

U. Draeger* and H.-J. Hohorst[3,4]

SUMMARY

The permeation kinetics of cyclophosphamide and its metabolites were studied with Ehrlich ascites tumor cells, murine L1210 leukemia cells, and mouse L929 fibroblasts at 1°C. In contrast to carboxyphosphamide and *nor*-nitrogen mustard, an equipartition of cyclophosphamide and 4-hydroperoxycyclophosphamide was observed in these cells. First experiments have been done to study the efflux of cyclophosphamide and 4-hydroperoxycyclophosphamide after incubation until saturation with Ehrlich ascites tumor cells. Cyclophosphamide could be washed out completely, whereas 4-hydroperoxycyclophosphamide shows an apparent retention in the cell at 37°C.

[Cancer Treat Rep 60:423-427, 1976]

For many reasons one can assume that an activated metabolite of an inactive anticancer agent must permeate into tumor cells to evolve its cytotoxic activity. More direct evidence for this concept would be obtained by a decrease in the cytotoxic activity of an impermeable protein-bound metabolite such as protein-bound 4-hydroxycyclophosphamide (4-hydroxy-CP).

It is possible that a different permeation of cytotoxic substances into tumor and normal cells may also influence the specificity of the compound. The permeation rate of an alkylating compound should be greater than the alkylation, which finally leads to the cytostatic activity. On the other hand the efflux of the substances should be slow enough that the metabolite can react with target molecules of the cell, and therefore influence cell division. To test these assumptions we have studied the permeation of cyclophosphamide (CP) and its metabolites into different tumor cells.

MATERIALS AND METHODS

CP was supplied by Asta-Werke AG, Brackwede, West Germany. ^{3}H-CP, uniformly labeled at the β-chloroethyl group, was obtained from Prof. Schaumlöffel (Klinik und Polyklinik für Nuclearmedizin, Marburg, West Germany). The specific activity was approximately 180 microcuries/μmol. 4-Hydroperoxycyclophosphamide (4-hydroperoxy-CP) was synthesized according to Takamizawa et al (1) and Peter et al (2). ^{3}H-4-hydroperoxy-CP, uniformly labeled at the β-chloroethyl group, was produced by the same method. The specific activity was 4 microcuries/μmol. Carboxyphosphamide and *nor*-nitrogen mustard were obtained from Asta-Werke AG. ^{3}H-labeled *nor*-nitrogen mustard was also obtained from Prof. Schaumlöffel. The specific activity was approximately 440 microcuries/μmol. 4-(4-Nitrobenzyl)pyridine (NBP) was purchased from EAG-Chemie, Steinbach, West Germany. 2-(4-*t*-Butylphenyl)-5-(4-biphenyl)-1,3,2-oxadiazole (butyl-PBD) was obtained from W. Zinsser, Frankfurt, West Germany. 1,3-Propanediol, cellulose plates for the thin-layer chromatography (TLC) testing, and silica gel plates (PSC-Fertigplatten Kieselgel 60 and DC-Fertigplatten Kieselgel 60) were obtained from E. Merck AG, Darmstadt, West Germany. Albumin from bovine serum was obtained from Serva, Heidelberg, West Germany. Silicone oil AR 20 was obtained from Wacker-Chemie GmbH, München, West Germany. ^{14}C-carboxydextran (with a radiochemical purity of 99%, a specific activity

[1]Supported by the Bundesministerium für Forschung und Technologie, Bonn, West Germany.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany.

[4]The technical assistance of Mrs. Xenia Klikic is gratefully acknowledged.

*Reprint requests to: Dr. U. Lenssen, Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, Theodor-Stern-Kai 7, 6000 Frankfurt 70, West Germany.

of 1.3 microcuries/mg, and a molecular weight of 60,000-90,000) and ^{3}H-labeled water (specific activity, 1 millicurie/g) were purchased from New England Nuclear, Langen, West Germany.

Cell material.—Ehrlich ascites tumor cells were routinely grown in NMRI mice (obtained from Versuchstierzucht G. Schwenke, Nauheim, West Germany) and collected 5-7 days after transplantation. They were washed and suspended in Krebs-Ringer phosphate buffer (pH 7.4) containing 1% bovine serum albumin. Murine L1210 leukemia cells were obtained from Dr. Drahovsky (Zentrum für Biologischen Chemie, University of Frankfurt, West Germany) and were suspended in RPMI medium (Flow Laboratories GmbH, Bonn, West Germany) containing 15% fetal serum. Mouse L929 fibroblasts were received from Mr. S. Kaul (Zentrum für Biologischen Chemie) and were suspended in EMEM medium (Flow Laboratories GmbH) containing 10% calf serum. The cells were counted in a Coulter Counter model B (Coulter Electronics, Hialeah, Fla) and were resuspended in the medium to about 10^7 cells/ml of medium.

NBP test.—The total concentration of the alkylating and potential alkylating compounds (defined as the alkylating capacity) in solution was determined according to Friedman and Boger (3) after a previous acid hydrolysis in 1 N HCl at 100°C for 10 minutes. All alkylating and potential alkylating compounds were determined by this method.

NBP test for the demonstration of alkylating material by TLC.—The cellulose plates were sprayed with 0.025 N acetate buffer (pH 4.6) and then with a solution of 2.5% NBP in acetone. The plates were heated for 10 minutes at 150°C and after cooling to room temperature they were sprayed with 0.3 N NaOH. Alkylating substances appeared blue stained.

Determination of peroxides.—Peroxides were determined on plates for TLC according to Stahl (4).

Measurement of radioactivity.—The radioactivity of solutions was determined in the liquid scintillation counter PW 4510/01 (Philips, Eindhoven, Netherlands) by adding 5-100 μl of the radioactive solution to 12 ml of scintillation liquid. The scintillation liquid contained 6 g of butyl-PBD in 600 ml toluene and 400 ml methylglycol. The radioactivity of compounds separated on cellulose plates by electrophoresis was measured in the scintillation counter after scraping the cellulose into a vial, adding the scintillation liquid, and mixing vigorously.

Purification of the ^{3}H-labeled substances.—^{3}H-CP and ^{3}H-carboxyphosphamide were purified on cellulose plates by TLC as described previously (5). The radioactive material, indicated on a reference strip, was scraped off and the compound was eluted at +4°C with dichloromethane. Ethanol was added and the dichloromethane removed under reduced pressure and at low temperature. Unpurified ^{3}H-carboxyphosphamide was obtained from the urine of rats after treatment with ^{3}H-CP. To separate most of the interfering material the urine was treated with cold ethanol at −20°C, and after evaporation of the ethanol the precipitate was removed by centrifugation. This procedure was repeated several times, and the supernatant was concentrated and used for the above-mentioned purification. ^{3}H-4-hydroperoxy-CP was purified on silica gel plates at −20°C with ethylacetate as running solvent. According to a reference strip, the silica gel was scraped off at −20°C and the compound was eluted with acetone at the same temperature. The solvent was carefully evaporated at −5°C to −10°C, and after adding 1-2 ml of ethanol the acetone was completely removed. ^{3}H-*nor*-nitrogen mustard was purified on cellulose plates by thin-layer electrophoresis (TLE) using a buffer (pH 6.5) of water:pyridine:glacial acetic acid (89:10:1). The TLE was run with a field strength of 27 volts/cm at 2°C for 30 minutes in the pherograph "Original Frankfurt" (Typ Mini 65; Hormuth und Vetter, Wiesbaden, West Germany). According to a reference strip the cellulose was scraped off and eluted with ethanol at +4°C. The solution was concentrated at a low temperature. The purity of the substances was checked using the methods of TLC and TLE described above. The specific radioactivity of the compounds was determined with the NBP test and by radioactivity measurements.

Protein determination.—The protein equivalent of the cell suspension was determined with the biuret method (6) after precipitation of the cells in 25% trichloroacetic acid. Before the optical density measurement the final samples were extracted with 2 ml diethyl-ether to remove lipids.

Silicone-oil filtration method.—The permeation kinetics were measured by the silicone-oil filtration method according to Pfaff and Klingenberg (7). A total of 30 μl of 12% perchloric acid was layered over by 70 μl of silicone oil AR 20, and then 100 μl of the cell suspension was added. After addition of 10 μl of a solution containing the ^{3}H-labeled compound (specific activity, 2 microcuries/μmol) and ^{14}C-dextran, the incubation was stopped by centrifugation in the Microfuge 152 (Beckman Instruments GmbH, München, West Germany). The acid layer with the cell pellet in each microcentrifuge tube was cut off and transferred into the vial, and after addition of the scintillation liquid the radioactivity was measured.

RESULTS AND DISCUSSION

The Pfaff-Klingenberg method couples the sedimentation of the cells with instantaneous deproteinization. The cells are spun down from their incubation medium through the silicone layer into 12% perchloric acid. Preliminary studies have shown that the maximum time needed by the cells to reach the deproteinizing layer was 2-3 seconds at 1°C. We assumed that this time was short enough with respect to the permeation kinetics and therefore could be ignored.

All apparent kinetics were measured at 1°C to prevent the breakdown and other reactions of the CP metabolites. Morphologic changes that might influence the permeability of the cells were checked by microscopy but were not observed at this temperature. In addition to the ^{3}H-labeled compounds, ^{14}C-dextran was added to the cell suspension to monitor the amount of medium that penetrated through the oil layer during the centrifugation.

The permeation kinetics of CP, 4-hydroperoxy-CP, carboxyphosphamide, and *nor*-nitrogen mustard in a final concentration of 1 mM were measured by this method. 4-Hydroperoxy-CP has been chosen as the only available radioactive "activated" CP metabolite; it is stable enough to allow the extensive purification needed for these experiments. All substances were incubated with Ehrlich ascites tumor cells in Krebs-Ringer phosphate buffer (pH 7.4) containing 1% bovine serum albumin (fig 1).

Significant differences can be observed in the apparent permeation rates as well as in the saturation kinetics. *Nor*-nitrogen mustard showed the greatest apparent uptake and reached its saturation after 20 minutes' incubation. In contrast to that, the anionic carboxyphosphamide showed very rapid kinetics, reaching saturation within about 20 seconds. The obviously lower apparent saturation value, if compared to the other substances, suggests that carboxyphosphamide was probably only adsorbed to the cell surface without permeating the membrane.

CP and 4-hydroperoxy-CP reached nearly the same saturation value, which was significantly lower than that of *nor*-nitrogen mustard. However, the peroxide showed the slowest kinetics. Since 4-hydroperoxy-CP and 4-hydroxy-CP evidently have similar physicochemical properties, as determined by their R_f values on TLC, we assume that 4-hydroxy-CP may penetrate in a fashion similar to that of 4-hydroperoxy-CP (shown in fig 1).

Further studies of the permeation of CP and 4-hydroperoxy-CP into other tumor cells, such as murine L1210 leukemia cells and mouse L929 fibroblasts, are presented in figure 2. The kinetics were measured under the same conditions used for the Ehrlich ascites tumor cells. We found no significant differences in the kinetics and the saturation

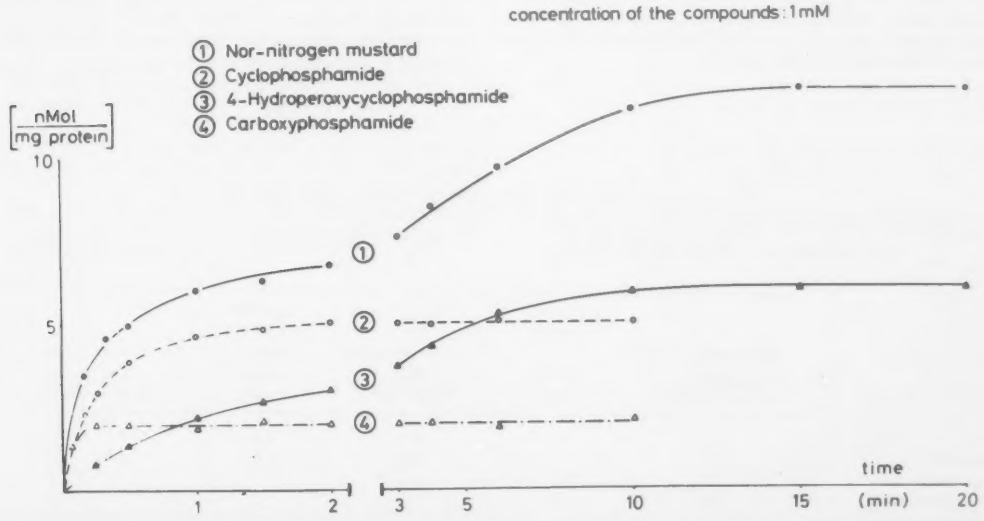


FIGURE 1.—Permeation of CP, its derivatives, and *nor*-nitrogen mustard into Ehrlich ascites tumor cells at 1°C. The compounds (concentration, 1 mM) were incubated with Ehrlich ascites tumor cells in Krebs-Ringer phosphate buffer (pH 7.4) containing 1% bovine serum albumin at 1°C. Kinetics were studied with the silicone-oil filtration method (*see* Materials and Methods).

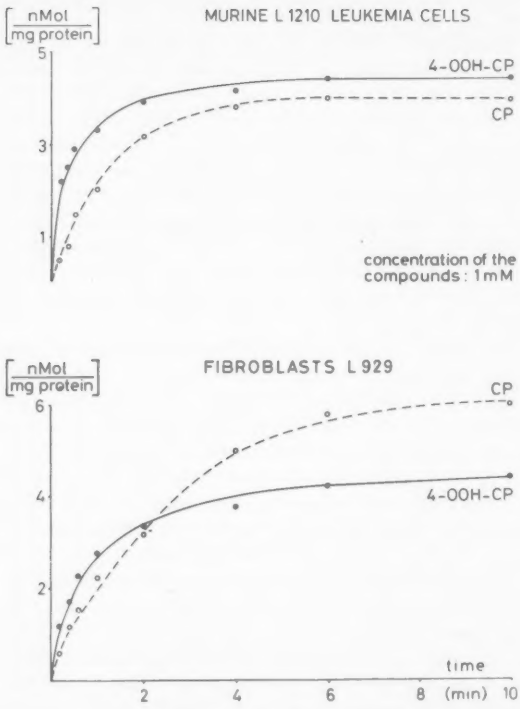


FIGURE 2.—Permeation of CP and 4-hydroperoxy-CP (4-OOH-CP) into murine L1210 leukemia cells and mouse L929 fibroblasts. The compounds (concentration, 1 mM) were incubated with murine L1210 leukemia cells suspended in RPMI medium containing 15% fetal serum (*top*) and with mouse L929 fibroblasts suspended in EMEM medium containing 10% calf serum (*bottom*) at 1°C. Kinetics were studied with the silicone-oil filtration method (*see* Materials and Methods).

values in comparison to the Ehrlich ascites tumor cells.

The saturation values and apparent cellular concentrations of the substances after the incubation with the different mouse cells at 1°C are given in table 1. The apparent cellular concentrations were calculated from the cell volume, determined with ^{3}H-labeled water by the silicone-oil filtration method. The table shows an approximate equipartition of CP and 4-hydroperoxy-CP in Ehrlich ascites cells (starting concentrations of 1 mM). In contrast, carboxyphosphamide shows a lower intracellular concentration. This is consistent with the suggestion that the compound is probably only adsorbed at the cell surface, as indicated by the rapid saturation kinetics described above. The reason for the higher, apparently cellular concentration of the cationic *nor*-nitrogen mustard may be additional adsorption since alkylation can be excluded. The same equipartition of CP and 4-hydroperoxy-CP can be observed in the other cell types.

First experiments have been done with Ehrlich ascites tumor cells to study the efflux of CP and 4-hydroperoxy-CP after incubation until saturation. For these studies the cells were incubated with 1 mM ^{3}H-CP or ^{3}H-4-hydroperoxy-CP in Krebs-Ringer phosphate buffer (pH 7.4) containing 1% bovine serum albumin. The incubation was carried out until saturation of the compound in the cell was reached, as assumed from the permeation kinetics. After centrifugation and drawing off the supernatant, the cells were resuspended several times with the same volume of buffer at 0°C. Each resuspension was for the same duration as the initial incubation. To destroy the cell membrane, 12% per-

TABLE 1.—Saturation values and apparent cellular concentrations*

Compound	Ehrlich ascites tumor cells		Murine L1210 leukemia cells		Mice L929 fibroblasts	
	Saturation value [nmol / mg protein]	Apparent cellular concentration [mM]	Saturation value [nmol / mg protein]	Apparent cellular concentration [mM]	Saturation value [nmol / mg protein]	Apparent cellular concentration [mM]
CP	5	1.5	4	1.5	6	1.3
4-Hydroxy-CP	6	1.7	4.5	1.6	4.5	1.2
Carboxyphosphamide	2.1	0.5				
Nor-nitrogen mustard	12.5	2.6				

*Saturation values were obtained from the permeation kinetics (figs 1 and 2). The apparent cellular concentrations were calculated from the saturation values and the cell volume, determined with ^{3}H-H_2O by the silicone-oil filtration method (*see* Materials and Methods). Initial extracellular concentration was 1 mM.

chloric acid was added to the cells after the last washing and the suspension was transferred into a vial for radioactivity measurements.

The decrease of the radioactivity after each resuspension at 0°C is shown in figure 3. Apparently CP could be washed out completely following incubation at 0°C and 37°C. In contrast to CP, the peroxide shows a remarkable difference in the apparent efflux after the incubations at 0°C and 37°C. It is questionable whether the small retention of the peroxide at 0°C is real or is due to an incomplete efflux after washing four times. This may be related to the slow permeation kinetics (fig 1). The obviously stronger retention of the peroxide during 37°C incubation suggests binding to the cell.

Evidently the retention does not result from an alkylation of the cells by the peroxide or by 4-hydroxy-CP which is released by spontaneous breakdown of 4-hydroperoxy-CP (5). As previously shown (5), the half-life of the alkylation of 4-hydroperoxy-CP was found to be 120 minutes in 0.07 M phosphate buffer (pH 7) at 37°C but 310 minutes in Krebs-Ringer phosphate buffer (pH 7). The half-life in Krebs-Ringer phosphate buffer may be the maximum half-life under physiologic conditions. Thus, another reaction is probably involved in the binding of the 4-hydroperoxy-CP, since physicochemical binding can be excluded, as we have shown by the CP experiment. On the basis of our studies of the reaction of 4-hydroxy-CP and the sulfhydryl compounds and their kinetics (8), the retention of 4-hydroperoxy-CP in the cells may be due to a reaction between protein and sulfhydryl groups and 4-hydroxy-CP, released from the peroxide.

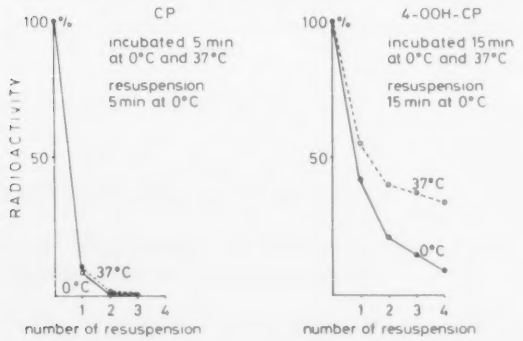


FIGURE 3.—Efflux of CP and 4-hydroperoxy-CP (4-OOH-CP) after saturation of the compounds with Ehrlich ascites tumor cells in Krebs-Ringer phosphate buffer (pH 7.4). ^{3}H-CP and ^{3}H-4-hydroperoxy-CP (concentration, 1 mM) were incubated with Ehrlich ascites tumor cells in Krebs-Ringer phosphate buffer (pH 7.4) containing 1% bovine serum albumin at 0°C and 37°C. CP was incubated with the cells for 5 mins; 4-hydroperoxy-CP was incubated for 15 mins. These times required to saturate the cells with the compound were assumed from the permeation kinetics. After centrifugation and drawing off the supernatant, the cells were resuspended several times with the same volume of buffer at 0°C. The radioactivity after saturation was set at 100% and the remaining activity after each resuspension was calculated relative to this.

REFERENCES

1. TAKAMIZAWA A, MATSUMOTO S, IWATA T, ET AL. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
2. PETER G, WAGNER T, and HOHORST H-J. Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. Cancer Treat Rep 60:429-435, 1976.
3. FRIEDMAN OM, and BOGER E. Colorimetric estimation of nitrogen mustard in aqueous media. Anal Chem 33:906-910, 1961.
4. STAHL E. Dünnschicht-Chromatographie, 2nd ed. Berlin, Springer-Verlag, 1967, p 836.
5. VOELCKER G, DRAEGER U, PETER G, ET AL. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
6. GORNALL AG, BARDAWILL CS, and DAVID MM. Determination of serum proteins by means of biuret reaction. J Biol Chem 177:751-766, 1949.
7. PFAFF E, and KLINGENBERG M. Adenine nucleotide translocation of mitochondria. 1. Specificity and control. Eur J Biochem 6:66-79, 1968.
8. DRAEGER U, PETER G, and HOHORST H-J. Deactivation of cyclophosphamide (NSC-26271) metabolites by sulfhydryl compounds. Cancer Treat Rep 60:355-359, 1976.

Studies on 4-Hydroperoxycyclophosphamide (NSC-181815): A Simple Preparation Method and its Application for the Synthesis of a New Class of "Activated" Sulfur-Containing Cyclophosphamide (NSC-26271) Derivatives[1,2]

G. Peter,* T. Wagner, and H.-J. Hohorst[3,4]

SUMMARY

4-Hydroperoxycyclophosphamide was obtained in approximately 20% yield by ozonization of cyclophosphamide in acetone/water at 0°C. It was reduced to 4-hydroxycyclophosphamide, which was treated with several mercaptans to yield compounds of the type 4-(*S*-*R*)-mercapto-cyclophosphamide. In the solid state these compounds are stable at room temperature; in aqueous solution they are hydrolyzed to 4-hydroxycyclophosphamide or its tautomer, aldophosphamide. One of the 4-(*S*-*R*)-mercapto-cyclophosphamide compounds was tested biologically in vitro against Yoshida ascites tumor cells and showed the same cytotoxic activity as 4-hydroxycyclophosphamide.

[Cancer Treat Rep 60:429-435, 1976]

MATERIALS AND METHODS

Novel Preparation of 4-Hydroperoxycyclophosphamide (4-Hydroperoxy-CP)

4-Hydroperoxy-CP was obtained by ozonization of 30 g of cyclophosphamide (CP) in a mixture of 120 ml of water, 60 ml of acetone, and 20 ml of 33% hydrogen peroxide at 0°C. Ozone/O_2 was bubbled through the solution with 45 mg O_3/minute[5] over 6 hours. CP reacted nearly quantitatively to yield approximately 50% 4-ketocyclophosphamide (4-keto-CP), approximately 20% 4-hydroperoxy-CP, and approximately 20% of its suggested diastereomer, shown by thin-layer chromatography (TLC) (silica gel,[6] ethyl acetate, −25°C [R_f of the 4-hydroperoxy-CP = 0.56; R_f of the 4-hydroperoxy-CP diastereomer = 0.07; R_f of the 4-keto-CP = 0.32]) and by radio-scanning the side-chain ^{3}H-labeled compounds.[7]

Acetone was evaporated at 0°C. After standing at 4°C for about 2 hours most of the 4-keto-CP had crystallized and was filtered off. The filtrate was extracted with 4 × 300 ml CH_2Cl_2. The collected organic layers were dried over Na_2SO_4 and concentrated to an oily residue, which was dissolved in 50 ml CH_2Cl_2 and stored at −20°C overnight. The crystallized 4-keto-CP was filtered off and washed with CH_2Cl_2 (−25°C). The filtrate was again concentrated to an oily residue and dissolved in 3 ml of CH_2Cl_2. Portion-wise addition of 30 ml of diethyl ether gave, while shaking and scratching the flask with a glass rod, after 1 hour, approximately 6 g of 4-hydroperoxy-CP, still contaminated with 4-keto-CP. For recrystallization the product was dissolved in boiling chloroform. After cooling to room temperature residual 4-keto-CP was filtered off. At −25°C 4-hydroperoxy-CP, was recrystallized and was shown by TLC to be the fast-running diastereomer.

[1]Supported by Bundesministerium für Forschung und Technologie.

[2]4-Hydroperoxycyclophosphamide: CAS reg. No. 39800-16-3; hydroperoxide, [2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorin-4-yl], *P*-oxide.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate. Supplied as a gift by Asta-Chemie, Brackwede, West Germany.

[3]Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, West Germany.

[4]We wish to thank Mrs. Xenia Klikic for her excellent assistance.

[5]Labor Ozoneur, Type Lab-50-1; Gebr. Herrmann, Köln-Ehrenfeld, West Germany.

*Reprint requests to: Dipl. Chem. G. Peter, Gustav-Embden-Zentrum der Biologischen Chemie, University of Frankfurt, Theodor-Stern-Kai 7, 6000 Frankfurt 70, West Germany.

[6]DC-Alufolien, Kieselgel 60, F_{254}, Art. 5554; Merck, Darmstadt, West Germany.

[7]Berthold-Radiochromatogram-scanner II, LB 2722.

To prepare 4-hydroperoxy-CP of high purity and with quantitative recovery (eg, for working with radioactive material), purification was carried out by preparative layer chromatography (PLC). Silica gel plates[8] were developed in ethyl acetate at −25°C. The peroxydic compounds were detected by spraying indicator strips with a spray for peroxides (1). The zone containing the product was removed, eluted with acetone at 4°C, and dried over Na_2SO_4.

^{3}H-4-Hydroperoxy-CP with a specific activity of 4 millicuries/mmol was prepared by the new method, starting from CP (specific activity 200 millicuries/mmol,[9] ^{3}H-labeled uniformly on the β-chloroethyl groups).

Synthesis of 4-(*S-R*)-mercapto-CP via 4-Hydroxycyclophosphamide (4-Hydroxy-CP) From 4-Hydroperoxy-CP

After reduction of 2 mmols of 4-hydroperoxy-CP to 4-hydroxy-CP with 2 mmols of triphenylphosphine (2), the CH_2Cl_2 extract was evaporated at 0°C and the residue dissolved in 1 ml of dry acetone or ethyl acetate. Four mmols of mercaptan was added and the mixture was stirred overnight at 4°C. Products with ethylmercaptan, 2-mercaptoethanol, tert. butylmercaptan, and benzylmercaptan were prepared. TLC (silica gel, ethyl acetate, room temperature) of each reaction mixture showed a slow- and fast-running product, which reacted with both 4-(4-nitrobenzyl)pyridine (NBP) (3) and sulfur spray (1).

After evaporation of the solvent to 200 μl, the products were isolated by PLC on silica gel with ethyl acetate at 4°C. In the case of the mercaptoethanol reaction product, ethyl acetate/acetone (4:1, vol/vol) was used. Extended chromatography was sometimes necessary to obtain satisfactory separation. This was achieved by evaporation of the solvent by warming[10] the front of the plate over 1-3 hours after the solvent front had reached it. All products were eluted from silica gel with acetone. After drying over Na_2SO_4 the solvent was evaporated completely. The residues were dissolved in 500 μl of diethyl ether (except the mercaptoethanol reaction product) and cooled on dry ice. Petroleum ether (boiling point 40°C-60°C) was then added dropwise until cloudiness appeared.

The flask was placed on dry ice overnight to yield crystalline products. The crystals were separated at −25°C and washed with dry ice-cooled petroleum ether. The yields of the fast-running diastereomers were 50%-80%.

The mercaptoethanol reaction product was dissolved after evaporation of acetone in chloroform and crystallized using the same procedure with diethyl ether on dry ice. The crystals melted during the filtration process in a −25°C cooling cell. Also the crystal of the benzylmercaptan product melted during the same procedure in the cooling cell to give a colorless oil which was crystallizable with water or by contact with air moisture.

Identification of the Compounds

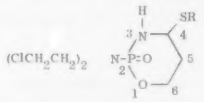
4-Hydroperoxy-CP

Ozonization of CP and fractional crystallization yielded the TLC fast-running diastereomer of 4-hydroperoxy-CP, which was identical with the product obtained by Takamizawa's synthesis (2). The identity was shown by proton magnetic resonance (PMR)[11] and infrared (IR)[12] spectrometry, TLC in different solvents, and the constant melting point (mp) of the mixture of the two compounds, prepared in different ways. After H/D exchange, PMR showed, with dimethylsulfoxide (DMSO-d_6) as solvent and tetramethylsilane (TMS) as internal reference, a vicinal coupling constant of the C-4 proton and C-5 protons of $J_{C\text{-}4\text{-}H,C\text{-}5\text{-}H_2} \sim 4$ Hz.

The slow-running compound was identified by TLC and sprays as a peroxide and alkylator, which converted partially in CH_2Cl_2 at −25°C to 4-hydroperoxy-CP, and was suggested to be a 4-hydroperoxy-CP diastereomer. It was separated by PLC at −25°C, eluted with acetone, and dried over Na_2SO_4; it showed significantly less stability than the fast diastereomer. Thus, a clear identification by means of spectral analysis has failed up to now.

4-(S-R)-mercapto-CP

The following outlines the identification of compounds of the type shown below.



I = R_1 = CH_2CH_3
II = R_2 = $C(CH_3)_3$
III = R_3 = CH_2CH_2OH
IV = R_4 = $CH_2C_6H_5$

[8]PSC-Fertigplatten, Kieselgel 60; Art. 5745, Merck, Darmstadt, West Germany.

[9]We are indebted to Prof. Schaumlöffel, Klinik und Poliklinik für Nuklearmedizin, Marburg/Lahn, West Germany, for supplying this sample.

[10]Camag Vario Heizung, step 1.

[11]We thank Dipl. Chem. M. Molter, Institut für Organische Chemie II, Frankfurt, West Germany, for running the PMR spectra and for stimulating discussions. All spectra determined with Bruker HX 90.

[12]Perkin Elmer 257 and 735.

I: 2-[bis(2-chloroethyl)amino]-4-mercaptoethyltetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide [4-(*S*-ethyl)-mercapto-CP], fast diastereomer, crystallized in white needles, mp 37°C-38°C,[13] stable at 4°C, soluble in CH_2Cl_2, $CHCl_3$, acetone, DMSO, and H_2O. IR^{KBr}_{max} (cm^{-1}): 3465, 3205 (NH), 2955, 1640, 1455,1260, 1215 (P = O), 1190, 1125, 1090, 1065 (P−O−C), 985, 935, 880, 745 (C−Cl). Fourier transformation (FT)-PMR analysis in DMSO-d_6 with TMS as internal reference gave the following results with suggested assignments: δ[14] 1.19 (3H, triplet, J = 7.3 Hz, $S-CH_2CH_3$), δ 1.5-2.5 (2H, multiplet, $C-5-H_2$), δ 2.59 (2H, quartet, $S-CH_2-$), δ 3.0-3.8 (8H, multiplet, $ClCH_2CH_2-$), δ 3.8-4.8 (2H, multiplet, $C-6-H_2$), δ 4.63 (1H, doublet of multiplet, $J_{H,P}$ = 21 Hz, $J_{H,C-5-H_2}$ = 4 Hz, $J_{H,NH}$ = 4.7 Hz, C−4−H), δ 5.41 (1H, doublet of doublet, $J_{H,P}$ = 6.7 Hz, $J_{H,CH}$ = 4.7 Hz, NH). Assignments of δ 2.59 and δ 1.19 were confirmed by spin decoupling. Addition of D_2O resulted in the disappearance of band δ 5.41 and showed the coupling constant of the C-4 proton and C-5 protons of $J_{C-4-H,\ C-5-H_2}$ = 4 Hz. Integration also was made in continuous wave (CW) mode in two solvents, acetone-d_6 (fig 1) and DMSO-d_6, and confirmed the assignments. TLC:silica gel, ethyl acetate, room temperature. R_f = 0.28 (the slow diastereomer R_f = 0.08). Mass spectra were run (4). The slow diastereomer was isolated on PLC and handled continuously at 4°C, because it was observed to have significantly lower stability than the fast one. It was analyzed by mass spectrometry as an eluate in acetone. The spectrum was identical with that of the fast diastereomer (4).

II: 2-[bis(2-chloroethyl)amino]-4-mercapto-tert.butyltetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide [4-(*S*-tert.butyl)-mercapto-CP], fast diastereomer, crystallized in clusters of colorless platelets, mp 86°C-88°C, stable at room temperature, soluble in CH_2Cl_2, acetone, DMSO, and H_2O (slightly). IR^{KBr}_{max} (cm^{-1}): 3410, 3240 (NH), 2960, 1450, 1405, 1270, 1235 (P = O), 1125, 1045 P−O−C), 980, 900, 745 (C−Cl). FT-PMR analysis in acetone-d_6 with TMS gave the following results with suggested assignments: δ 1.37 (9H, singlet, $C(CH_3)_3$), δ 2.0-2.4 (2H, multiplet, $C-5-H_2$), δ 3.2-3.9 (8H, multiplet, $ClCH_2CH_2$), δ 4.0-4.8 (2H, multiplet, $C-6-H_2$), δ 4.8 (2H, broad doublet, $J_{H,P}$ = 18 Hz, C−4−H and NH). The band of NH is under the band δ 4.8. Mass spectra were run (4). TLC: silica gel, ethyl acetate, room temperature. R_f = 0.36 (the slow diastereomer R_f = 0.11).

[13]All melting points are uncorrected and were determined on a copper block.

[14]Chemical shift in ppm.

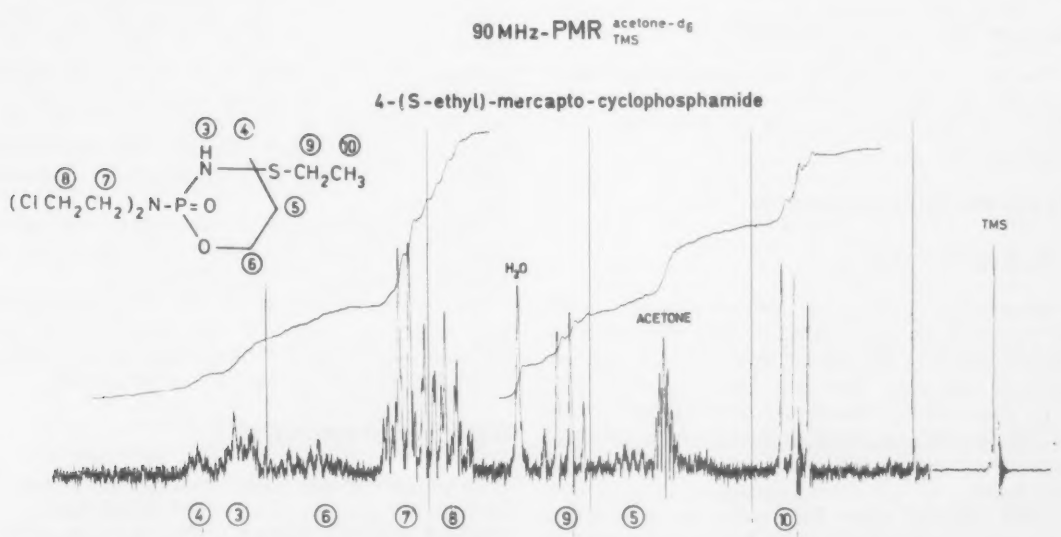


FIGURE 1.—10 = δ1.23, 3H; 5 = δ 2.11-2.47, 2H; 9 = δ2.67, 2H; 7 and 8 = δ 3.21-3.83, 8H; 6 = δ 3.83-4.44, 2H; 3 and 4 = δ 4.44-5.03, 2H. CW-PMR spectrum of 4-(*S*-ethyl)-mercapto-CP with solvent acetone-d_6 was chosen for demonstration, because of the better integration (compared with the FT mode) and to show the signals of the substituent protons (9 and 10). Using DMSO-d_6 the $S-CH_2$ signal (9) is partially superimposed by DMSO. Unfortunately, in acetone-d_6 the signal of NH falls together with the C−4−H signal, showing by integration 2 protons. In DMSO-d_6 the NH signal is well separated as doublet of doublet. The $C-5-H_2$ signal is overlapped by acetone, which is an impurity of the solvent, as well as water.

III: **2-[bis(2-chloroethyl)amino]-4-mercaptoethanol-2-ol-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide** [4-(*S*-ethan-2-ol)-mercapto-CP], fast diastereomer, obtained as a colorless oil at room temperature, showed good solubility in water and acetone. IR_{max}^{film} (cm^{-1}): 3290 (very broad; OH, NH), 2960, 1240 (P = O), 1140, 1075 (P–O–C), 1000, 960, 765 (C–Cl). TLC: silica gel, ethyl acetate, acetone (4:1, vol/vol), room temperature. R_f = 0.21 (the slow diastereomer R_f = 0). Further analysis has not yet been done.

IV: **2-[bis(2-chloroethyl)amino]-4-mercaptobenzyl-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide** [4-(*S*-benzyl)-mercapto-CP], fast diastereomer, obtained as white crystals, mp 64°C, stable at room temperature, soluble in acetone and H_2O (very slightly). IR_{max}^{KBr} (cm^{-1}): 3165 (NH), 2890, 1600, 1490 (aryl-H), 1440, 1365, 1305, 1260, 1235, 1195, 1100, 1060, (P–O–C), 990, 975, 950, 895, 775 (C–Cl), 735 (benzene, nonsubstituted), 710. Mass spectra were run (4). TLC: silica gel, ethyl acetate, room temperature. R_f = 0.32 (the slow diastereomer R_f = 0.12). The slow diastereomer, isolated as PLC eluate in acetone, showed a lower stability than the fast one (*see also* compound I). This compound was probably partially decomposed when analyzed by field desorption mass spectrometry; the peak of the molecular ion had a low intensity compared with other peaks (4).

Biologic Test of 4-(*S*-ethyl)-mercapto-CP

The in vitro assay of the cytotoxic activity against isolated Yoshida ascites tumor cells from rats was performed according to Schmähl and Druckrey (5).

RESULTS AND DISCUSSION

4-Hydroperoxy-CP

Ozonization of CP in acetone/water at 4°C yielded 4-keto-CP in approximately 50% yield and the diastereomers of 4-hydroperoxy-CP both in approximately 40% yield. The fast diastereomer in TLC was obtained by fractional crystallization and shown by IR and PMR analysis to be identical with 4-hydroperoxy-CP obtained by Takamizawa's synthesis.

PMR showed, after H/D exchange, a coupling constant of the C-4 proton and C-5 protons of $J_{C-4-H,C-5-H_2}$=4 Hz. Presuming the oxazaphosphorine ring to have the chair form (6), this result points to an axial position of the hydroperoxy group. The corresponding coupling constant of the equatorial-substituted isomer is expected to be in the range of J = 12 Hz (7). Unfortunately this could not be demonstrated due to the low stability of the suggested compound. According to the phosphorus substituents, the axial oxygen-equatorial dialkylamino group configuration was found for CP to be the stable one in solution and in solid state (8). By analogy with this, probably only two stable diastereomeric C-4-substituted CP derivatives are expected, resulting from the C-4 asymmetry.

The formation of 4-keto-CP and the 1:1 ratio (approximately) of 4-keto-CP to 4-hydroperoxy-CP (including its probable diastereomer) suggests the following rationalization of the reaction:

1. CP + O_3 ⟶ 4-keto-CP + $^1O_2$ (singlet O_2)
2. CP + $^1O_2$ ⟶ 4-hydroperoxy-CP

Reduction of ozone by phosphines, phosphide esters, sulfides, and even hydrocarbons, to produce singlet O_2 and the corresponding oxidized products is known (9), as well as the formation of hydroperoxides by insertion reactions of singlet O_2 into C–H bonds, eg, autoxidation of fatty acids (10). The direct oxidation of CP to yield 4-hydroperoxy-CP cannot be explained by attack of oxygen in ground state because CP and 4-hydroperoxy-CP are in singlet state while O_2 is in triplet state. Wigner's rules forbid reactions in which spin is not conserved (11). Moreover, the reaction must be endothermic. Spin and energy barriers could be overcome when singlet O_2 was the active species.

The probability of this assumption was shown by an experiment in which the solution of CP in acetone/water and H_2O_2 was treated with sodium hypochlorite forming singlet O_2 with H_2O_2 (12). $^1O_2$ reacted with CP to 4-hydroperoxy-CP. This was found in TLC in a small amount along with a large amount of CP. No other alkylating compounds were detectable.

Further experiments to investigate the mechanism of the reaction described and to eliminate the formation of 4-keto-CP are in progress.

This new method allows a fast and easy preparation of 4-hydroperoxy-CP and 4-hydroxy-CP, also as radioisotope-labeled compounds, when starting from commercially available radioactive CP.

4-(*S*-*R*)-mercapto-CP

To prepare a new class of sulfur-containing CP derivatives via 4-hydroxy-CP, 4-hydroperoxy-CP was the starting material. The chromatographically fast diastereomer of most of these derivatives had in solid state a significantly higher chemical stability than 4-hydroperoxy-CP. After reduction of 4-hydroperoxy-CP with triphenyl-phosphine we obtained 4-hydroxy-CP which was used, without

being isolated, to yield in acetone or ethyl acetate with sulfhydryl groups (RSH) compounds of the type 4-(*S-R*)-mercapto-CP. The reaction proceeded at 4°C overnight to yield the TLC fast diastereomer in 50%-80% yield, depending on the mercaptan used. The residue of alkylating compounds in the reaction mixture consisted of the slow diastereomer and of phosphoramide mustard [2-bis-(2-chloroethyl)phosphorodiamidic acid].

The PMR spectrum of the fast diastereomer of compound I protons showed after H/D exchange a coupling constant of the C-4 proton and C-5 protons of $J_{C-4-H,C-5-H_2}$ = 4 Hz; this diastereomer is suggested to be the *cis*-isomer ($-SCH_2CH_3$ is in the axial position and the exocyclic amido group in the equatorial position), based on the interpretation above.

If we used the reaction mixture containing trichloroacetic acid in 1% of the solvent volume and the pure fast diastereomer of 4-hydroxy-CP, the yield was 97% after 15 minutes at 0°C and only the fast diastereomer of the 4-mercapto-CP product was found. We therefore assume that 4-hydroxy-CP undergoes reaction, favored by acid catalysts, directly with the nucleophilic sulfhydryl (SH) group with a rate higher than the competing equilibration to tautomeric aldophosphamide, since aldophosphamide can, via re-equilibration, form the two diastereomers of 4-hydroxy-CP and thus also the two corresponding diastereomeric 4-mercapto-CP compounds (fig 2, reaction scheme 1, upper part).

The observation that the chromatographically pure fast 4-hydroxy-CP diastereomer yielded both diastereomeric 4-mercapto-CP derivatives reacting in the absence of acid over 12 hours at 4°C can be interpreted in the following ways:

a. Due to the long reaction time, aldophosphamide can be formed, which reconverts to the two diastereomers of 4-hydroxy-CP and reacts with SH groups (fig 2, reaction scheme 1, lower part).

b. After ring opening of 4-hydroxy-CP to aldophosphamide, the carbonyl group reacts with the SH group to form an intermediate semimercaptal and then recombines to the oxazaphosphorine ring, yielding the two diastereomers of the 4-mercapto-CP product (fig 2, reaction scheme 2).

The latter pathway is supported by the finding that aldophosphamide, isolated at 4°C on TLC (13), reacted with benzylmercaptan to yield products identical to those products which are found chromatographically when 4-hydroxy-CP was reacted with benzylmercaptan. Moreover, when 4-hydroxy-CP was reacted with 1,3-propanedithiol, in the absence of acid, aldophosphamide-trimethylenedithioacetal is most probably formed as evidenced by mass spectrometry.

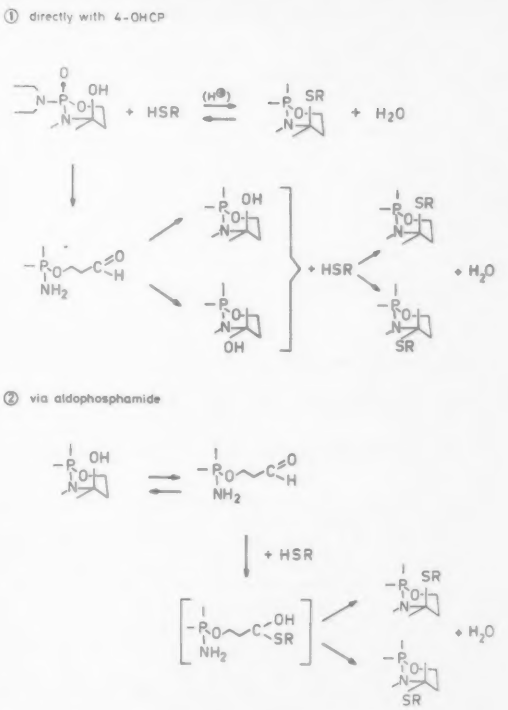


FIGURE 2.—For the reaction of thiols with 4-hydroxy-CP, 2 alternative pathways are discussed according to our observations: (1) direct reaction on 4-hydroxy (without intermediary ring opening), and (2) reaction with the tautomeric aldophosphamide to 4-(*S-R*)-mercapto-CP, which exists in diastereomers. In both cases the competing reaction is the tautomerism 4-hydroxy-CP/aldophosphamide. If only 1 of the 4-hydroxy-CP diastereomers is applied and the formation rate of 4-mercapto-CP is higher than the formation rate of aldophosphamide (oy acid catalysis), only 1 mercapto-CP diastereomer is found. Without acid catalysis the reaction rate of SH groups with the OH groups is slow and in competition with the equilibrium 4-hydroxy-CP/aldophosphamide to yield the 2 4-hydroxy-CP diastereomers which react with thiols to form the 2 diastereomeric products (pathway 1). In the alternative pathway (2) the tautomeric aldophosphamide reacts with thiols giving an intermediary semi-mercaptal, yielding after ring closure the 2 4-mercapto-CP diastereomers.

Hydrolysis of 4(*S-R*)-mercapto-CP

A 1.0 mM solution of compound I in 0.07 M phosphate buffer (pH 7) was incubated at 37°C. Samples were taken at different times and the concentration of alkylating species was measured by the NBP test after acid hydrolysis (fig 3, curve 2). Af-

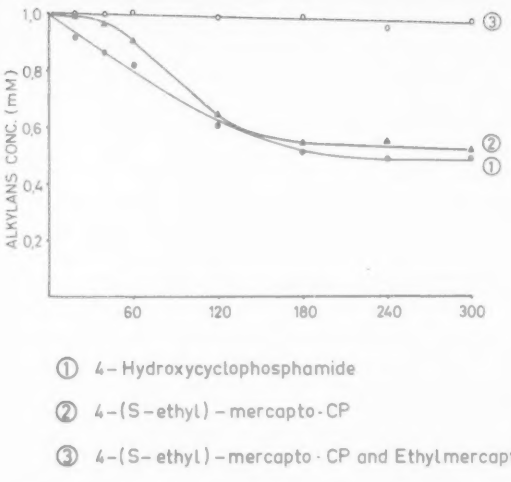


FIGURE 3.—Decrease of alkylator concentration in phosphate buffer (pH 7; 0.07 M; 37°C). 4-(*S*-ethyl)-mercapto-CP in 1.0 mM solution was incubated and the concentration of alkylating agents was measured at different times. The decrease of the concentration of alkylating agents (2) shows a behavior very similar to that of 4-hydroxy-CP (1) after a lag phase of 14 mins; 4-(*S*-tert.butyl)-mercapto-CP and 4-(*S*-ethan-2-ol)-mercapto-CP show similar behavior. The experiment was varied using separate portions of 500 μl of the 1.0 mM solution for each measure point and by addition of 100 μl of ethyl mercaptan to each test tube, closed during incubation; the initial concentration of alkylating agents was nearly constant over 5 hrs (3).

ter a lag phase of 15 minutes the decrease of alkylating species showed a behavior similar to that of 4-hydroxy-CP (fig 3, curve 1), treated under the same conditions, as well as compounds II and III. Moreover, phosphoramide mustard was found to be a product of incubation. Release of SH groups was shown with aldrithiol-4,[15] a reagent on SH groups. This behavior indicates that the 4-(*S*-*R*)-mercapto-CP derivatives are hydrolyzed to the mercaptans and to 4-hydroxy-CP or its tautomer, which is detectable after decomposition as the more stable phosphoramide mustard.

In subsequent incubation experiments the conditions described above were varied: for every measurement a separate portion of 500 μl of the 1.0-mm solution of compound I was used and incubated in closed test tubes containing 100 μl of additional ethylmercaptan.

This experiment showed the initial concentration of alkylating species to be nearly constant over several hours (fig 3, curve 3). This indicates that there might exist an equilibrium (14): 4-(*S*-ethyl)-mercapto-CP + $H_2O \rightleftharpoons$ 4-hydroxy-CP + HSR.

Biologic Test

Tests of the cytotoxic activity of 4-(*S*-ethyl)-mercapto-CP[16] have shown this compound to have almost the same cytotoxic activity in vitro as 4-hydroxy-CP (20 cytostatic units/μmol for both compounds). Further tests with the other 4-(*S*-*R*)-mercapto-CP derivatives are in progress.

The significantly higher chemical stability of the 4-(*S*-*R*)-mercapto-CP derivatives compared with 4-hydroxy-CP and 4-hydroperoxy-CP, as well as the possibility of decomposition in aqueous solution to cytotoxic active agents, may allow these 4-(*S*-*R*)-mercapto-CP compounds to be used in experimental studies, instead of activated CP, and for local application.

REFERENCES

1. STAHL E. Dünnschicht Chromatographie, 2nd ed. Berlin, Springer-Verlag, 1967, pp 835-836.
2. TAKAMIZAWA A, MATSUMOTO S, IWATA T, ET AL. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
3. FRIEDMAN OM, and BOGER E. Colorimetric estimation of nitrogen mustards in aqueous media. Anal Chem 33:906-910, 1961.
4. PRZYBYLSKI M, RINGSDORF H, VOELCKER G, ET AL. Characterization of cyclophosphamide (NSC-26271) metabolites and related derivatives by field desorption and electron-impact mass spectrometry. Cancer Treat Rep 60:509-516, 1976.
5. SCHMÄHL D, and DRUCKREY H. Contributions to the mode of action of *N*-oxide of nitrogen mustard. Naturwissenschaften 43:199-203, 1956.
6. CAMERMAN N, and CAMERMAN A. Cyclophosphamide structure. Molecular structure of 4-ketocyclophosphamide. J Am Chem Soc 95:5038-5041, 1973.
7. KARPLUS M. Vicinal proton coupling in nuclear magnetic resonance. J Am Chem Soc 85:2870-2871, 1963.
8. CLARDY JC, MOSBO JA, and VERKADE JG. Crystal and molecular structure of cyclophosphamide hydrate. JCS Chem Comm, 1972, pp 1163-1164.
9. MURRAY RW, LUMMA WC, JR. and LIN JWP. Singlet oxygen sources in ozone chemistry. Decomposition of oxygen-rich intermediates. J Am Chem Soc 92:3205-3207, 1970.
10. RAWLS R, and VAN SAMTEN PJ. A possible role of singlet oxygen in the initiation of fatty acid autoxidation. J Am Oil Chem Soc 47:121-125, 1970.

[15]4,4-Dithiodipyridine, Art. 14.305-7, Aldrich-Europe, Düsseldorf, West Germany (*see also* data sheet).

[16]We are obliged to Prof. N. Brock, Pharmakologische Abtlg. der Asta-Werke, Brackwede, West Germany, for determination of the in vitro activity.

11. WIGNER EP. Group Theory. New York, Academic Press, 1959.
12. FOOTE CS, WEXTER S, ANDO W, ET AL. Chemistry of singlet oxygen. IV. Oxygenations with hypochlorite-hydrogen peroxide. J Am Chem Soc 90:975-981, 1968.
13. VOELCKER G, DRAEGER U, PETER G, ET AL. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
14. DRAEGER U, PETER G, and HOHORST H-J. Deactivation of cyclophosphamide (NSC-26271) metabolites by sulfhydryl compounds. Cancer Treat Rep 60:355-359, 1976.

Studies on the Metabolism of Isophosphamide (NSC-109724) in Man[1]

K. Norpoth[2,*]

SUMMARY

A method is described for the quantitative determination of cyclophosphamide, isophosphamide, and trophosphamide and their stable alkylating metabolites on thin-layer chromatography plates with the aid of 4-pyridinealdehyde-2-benzothiazolylhydrazone (PBH). The method requires only 0.01-0.02 ml of urine from patients treated with high doses of cyclophosphamide or isophosphamide, but a careful standardization is required by using reference substances. In the urine of patients treated with isophosphamide, the 4-carboxy derivative and two different dechloroethyl derivatives resulting from side-chain oxidation are the predominant metabolites determined, next to isophosphamide. Side-chain oxidation may be prevalent. Determination of isophosphamide and its stable alkylating metabolites in the urine of 25 patients shows that the ratio of unchanged isophosphamide to its metabolites varies widely. The mean value of the total amount of PBH-reactive compounds exceeded 50% of the given dose, whereas < 1% was found to be converted to urine metabolites which release acrolein.

[Cancer Treat Rep 60:437-443, 1976]

The metabolism of cyclophosphamide (CP) as well as its analogs isophosphamide (IP) and trophosphamide is reported to differ widely from patient to patient (1,2). For elucidating this variability quantitative analytic procedures which allow the determination of single metabolites are more suitable than the usual method of determining all alkylating compounds using the NBP reaction or determining the total radioactivity in body fluids after injection of ^{3}H- or ^{14}C-labeled drugs. Differential metabolite analysis not only shows the extent of individual drug conversion but also identifies the predominant metabolic pathways in each case.

Sufficiently sensitive analytic procedures such as gas-chromatographic, infrared-spectroscopic, and mass-spectroscopic methods have been described for determination of CP and IP (3-9), but there is a need for methods to measure single metabolites. By means of biologic test methods toxic activities can be estimated in body fluids (10) but no differentiation between toxic agents is possible. In our experience, gas chromatography appears to be the most promising method. At present, however, we prefer the spectroscopic quantitation of alkylating urinary metabolites on thin-layer chromatography (TLC) plates after high-dose IP treatment. For this purpose the alkylating fractions are sprayed with 4-pyridinealdehyde-2-benzothiazolylhydrazone (PBH), a compound originally described as a useful chromogen for detecting alkylating agents by Sawicki and co-workers (11,12).

By the reaction shown in figure 1 on the TLC plate heated above hot acetophenone vapor to 180°C-200°C, stable red spots arise with an extinction maximum at 530 nm. Because the relationship between the extinction values and the quantities of the alkylating substances is not linear, a careful standardization of the analytic methods is needed.

Reproducible results can only be obtained with highly purified reagents and solvents. Exact quantitation requires the evaluation of extinction functions on each plate by applying a number of differently dosed samples of the substance in question to the same TLC plate. Experimental results from studies on IP metabolites show that standard deviations of $< \pm 3\%$ were not possible since the dependency of the extinction value on the amount of metabolite diverges increasingly from a linear

[1] Isophosphamide: CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide; ifosfamide. This agent is a test drug of the Asta-Werke AG, Brackwede, West Germany.

[2] Institüt für Staublungenforschung und Arbeitsmedizin, Universität Münster, West Germany.

*Reprint requests to: Dr. K. Norpoth, Institüt für Staublungenforschung und Arbeitsmedizin, Universität Münster, Westphalia, D 44 Münster, Westring 10, West Germany.

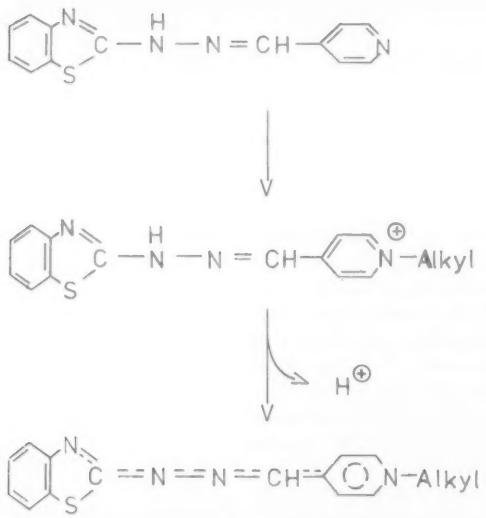


FIGURE 1.—PBH reaction with alkylating agents [after Sawicki et al (11,12)].

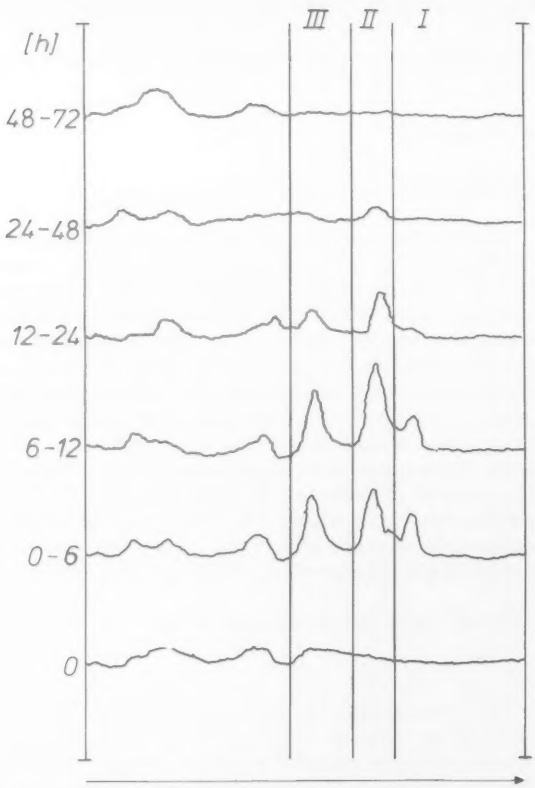


FIGURE 2.—TLC spectrograms of PBH-reactive urine components found at various time intervals after IP treatment.

relationship as the concentration rises. We started our investigations by identifying the alkylating fractions which can be colored by the PBH spray. For TLC we used Alufolien-Kieselgel (Merck AG, Darmstadt) and a chloroform/methanol/H_2SO_4 mixture (90:52:7.5) as solvent. Figure 2 shows TLC spectrograms obtained after application of 0.02 ml of urine from a patient who had been treated with 6 g of IP (infused in a 5% solution of levulose in 2 hours). Unconverted IP (peak I) could still be detected in the urine collected 12-24 hours after the start of the treatment. Peak II comprises two metabolites, the urinary concentration of which decreased insignificantly during the 24 hours following treatment. Peak III was carboxy-IP. We were able to separate and purify the metabolites in peak II. Elemental analysis showed a P:N:Cl ratio of 1:1:1. The mass-spectrometric analysis, performed with Dr. G. Müller at the Institute of Inorganic Chemistry of our University, suggested the existence of two different dechloroethylated products with the same molecular weight of 198/200. This was supported by further investigations with H. Raidt, which prove that in vitro oxidation of IP by means of $KMnO_4$ produces identical compounds. The same transformation of CP has been reported (13). Connors and co-workers described the dechloroethylated derivative of CP and an analog derivative of IP as products of the turnover with liver microsomes (14). Bakke and co-workers described dechloroethyl-CP as a minor metabolite in sheep (3). Therefore we were surprised to find that after treatment with IP the dechloroethylated metabolites provided the major part of the PBH-reactive urinary compounds. However, this is not always the case. Figure 3 shows the common excretion kinetics of both metabolites. The measured concentrations are exceeded clearly by those of carboxy-IP. No unconverted IP could be detected in the urine collected 12-24 hours after starting the treatment. The excretion rates shown in figure 4 may be regarded as an example of retarded biotransformation. More IP than IP metabolites was excreted. Interpretation of the values of metabolite concentrations from the urine analyses of five patients (shown in fig 5) must take into account the large biologic fluctuations of metabolic patterns. The excretion functions suggest that unconverted IP is excreted first, followed by carboxy-IP, whereas the elimination of the dechloroethyl derivatives lasted for a longer period. Figure 6 shows the excretion functions for a patient whose urine contained the highest concentrations of dechloroethyl deriva-

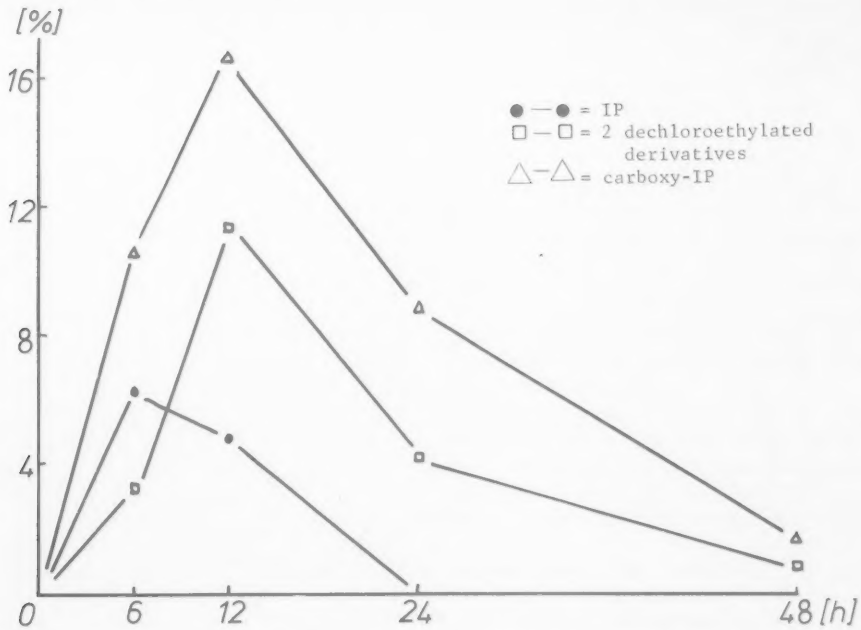


FIGURE 3.—Urine excretion of PBH-reactive compounds by a patient treated with 5 g of IP expressed as percent of the administered dose vs time.

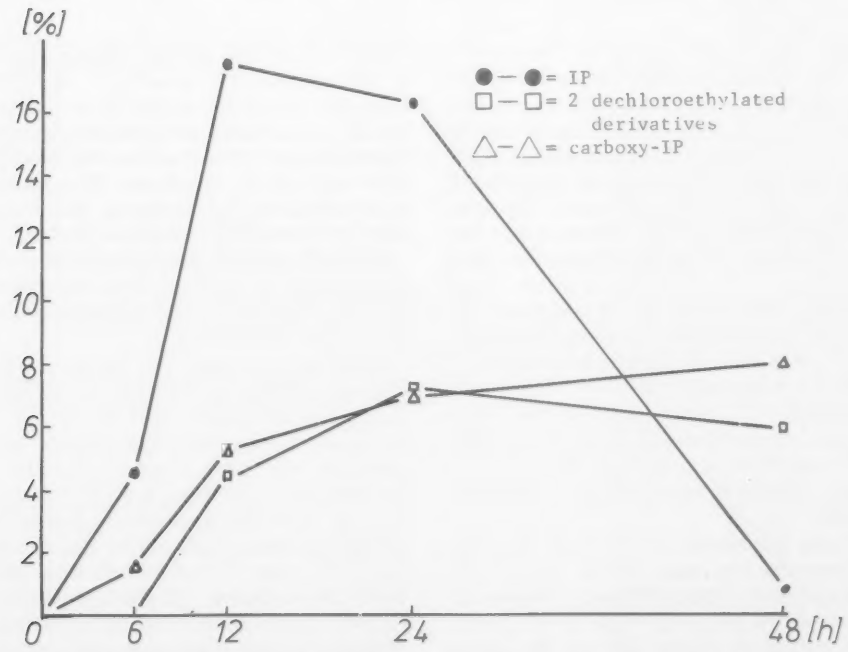


FIGURE 4.—Urine excretion of PBH-reactive compounds by a patient treated with 6 g of IP.

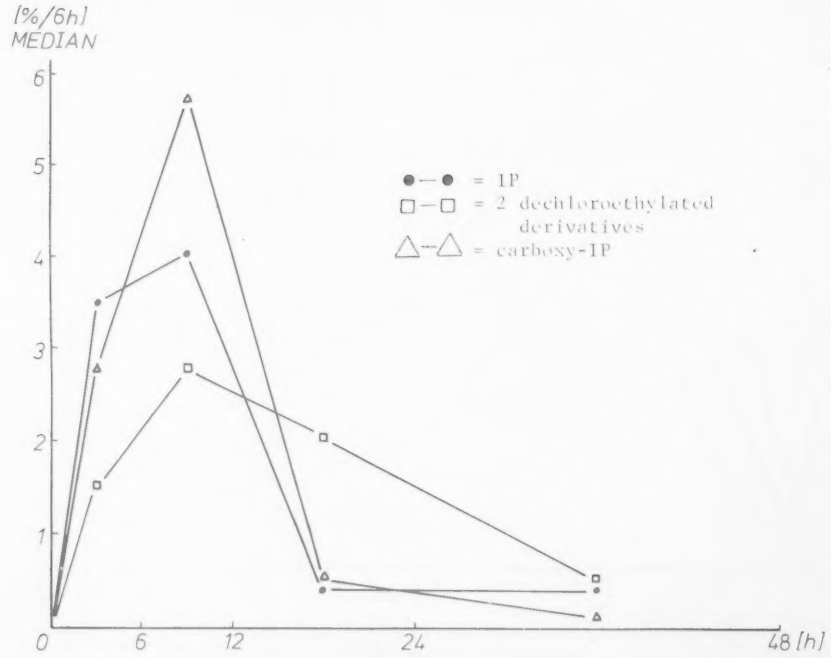


FIGURE 5.—Mean values of the urine excretion of PBH-reactive compounds calculated from the values of 5 patients treated with 5 g of IP.

tives yet found. The total amount of these compounds was 48% of the dose given. On the other hand, carboxy-IP and unconverted IP accounted for only 2.2% and 4.7% respectively. Since nearly the same values were found after a second infusion of 6 g of IP, this patient provided an example of major side-chain oxidation. Nevertheless, the parvicellular bronchus carcinoma of the patient was obviously in remission 2 weeks after the treatment.

Concerning the metabolism of trophosphamide, we conclude that by side-chain oxidation this drug forms metabolites which are identical to those of CP and IP (fig 7). Separating the main alkylating urinary metabolites of trophosphamide we found only one product which differed from all the metabolites of CP and IP with regard to its migration properties. This compound we assume to be carboxytrophosphamide.

In summarizing the results of 19 urine analyses after IP treatment, the mean value of the unchanged drug excreted within 48 hours corresponded to the reported value in the case of CP treatment (1), amounting to about 18% of the given dose. We found, however, a large standard deviation of about 70% of this value. In the urine of six of 25 patients the quantities of IP were higher than the quantities of the three main metabolites. In 14 cases more dechloroethylated derivatives were excreted than carboxy-IP, and in 11 cases more carboxy-IP was found. Thus, not only the ratio of unchanged to converted IP but also the ratio of ring to side-chain oxidation varied widely.

Further interindividual variations of IP metabolism seem to be caused by conjugation and alkylation reactions, especially those involving sulfur-containing compounds.

Speculations about the product split off by dechloroethylation of CP and its analogs have been presented by several authors (14,15). Whitehouse and co-workers assume that it should be chloroacetaldehyde and have demonstrated the immunosuppressive and cytotoxic activity of this compound (15). If chloroacetaldehyde is indeed the product, the metabolism of CP and its analogs partly corresponds to that of well-known toxic agents, eg, 1,-1,2-trichloroethane, 1,2-dichloroethane, chloroacetic acid (used as an alkylating herbicide), and vinylchloride. In vivo chloroacetaldehyde should be rapidly converted to chloroacetate. The metabolism of

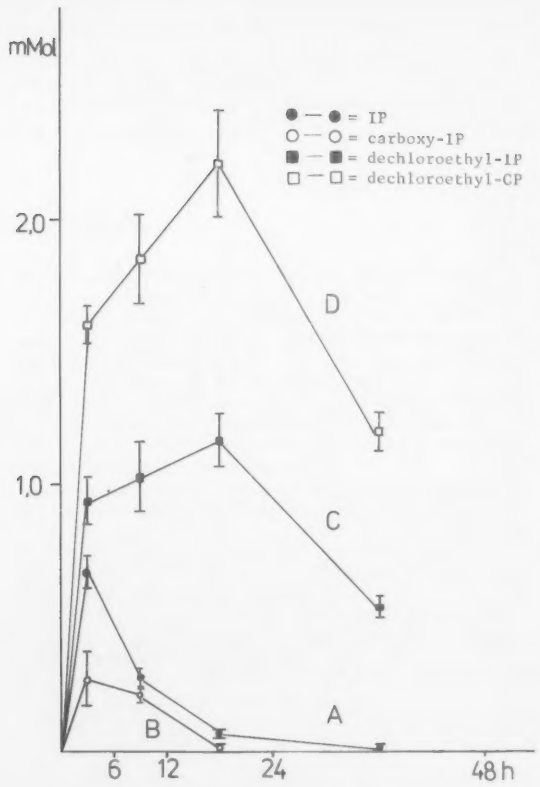


FIGURE 6.—Urine excretion of PBH-reactive compounds by a patient treated with 6 g of IP.

this compound in mice has been studied by Yllner (16,17) who found that only 6%-22% was eliminated unchanged by urine excretion.

The main urinary metabolites of chloroacetate were *S*-carboxymethylcysteine (33%-43% of the given dose) and thiodiacetic acid (33%-42%), whereas glycolic and oxalic acids were minor metabolites. Chloroacetaldehyde, however, may not only be transformed to chloroacetic acid but also into cysteinyl derivatives and into thioacetals, the cytotoxic properties of which are unknown. Regardless of whether chloroacetaldehyde or chloroacetic acid is the main breakdown product of IP (and also CP) dechloroethylation, the final steps of its metabolism can be regarded as clarified and are in accordance with the findings of Yllner (16). This is illustrated in figure 8. In our laboratory Dr. G. Müller has shown that *S*-carboxymethylcysteine and thiodiacetic acid were excreted in large amounts after IP infusion. *S*-carboxymethylcysteine was measured with an amino acid analyzer and thiodiacetic acid, as the dimethyl derivative, by capillary gas chromatography after methylation of urine samples.

Table 1 shows the results of an analysis of the urine of a patient treated with 6 g of IP. In this case the excretion was accelerated by simultaneous infusion of 4-chloro-*N*-(2-furfuryl)-5-sulfamoylanthranilic acid, a diuretic agent (20 mg).

As demonstrated by the values of acrolein-releasing metabolites, very small amounts of these products of ring oxidation have been found. This corresponds to the results of three other analyses. The total amount of acrolein-releasing metabolites was only about 1% of the given dose. The amount excreted did not decrease markedly after 24 hours. Thus it seems to be especially desirable to investigate the dose-dependent production and excretion of these derivatives and to compare the results, as well as the biologic variations, with those observed with other single metabolites.

By further determination of the excretion of IP as well as of single metabolites arising from ring and side-chain oxidation, and by further improvement of our analytic procedures, we expect to answer the following questions: (*a*) Can we demonstrate clear correlations between the curative and toxic effects of CP and its analogs and the preferred metabolic pathways? (*b*) Can we prove clear influences of certain accompanying treatments on

TABLE 1.—Urine excretion of IP and some of its metabolites with time in a patient treated with 6 g of IP

Time (hrs)	IP (mmol)	Carboxy-IP (mmol)	Dechloroethyl-IP I (mmol)	Dechloroethyl-IP II (mmol)	*S*-carboxymethylcysteine (mmol)	Thiodiacetic acid (mmol)	Acrolein-releasing metabolites (μmol)
6	0.106	0.28	0.11	0.62	—	0.02	0.6
12	0.019	trace	0.87	0.95	1.5	0.13	1.2
24	—	—	0.45	1.31	0.9	0.45	0.9
48	—	—	—	—	>0.05	0.30	1.0
72	—	—	—	—	trace	0.17	0.8
Total	0.125	0.28	1.43	2.88	2.4	1.07	4.5

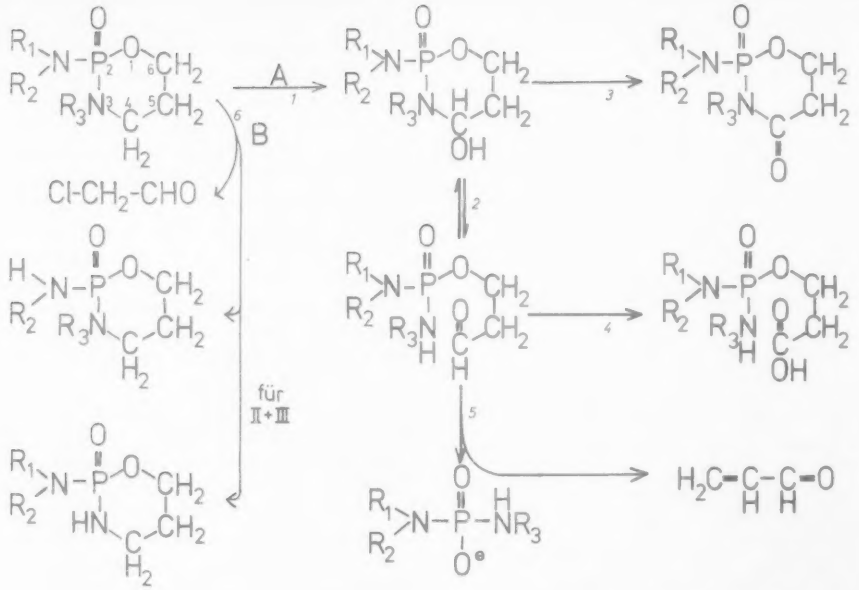


FIGURE 7.—Common pathways of CP, IP, and trophosphamide. I (CP): $R_1 = R_2 = Cl\text{-}CH_2\text{-}CH_2\text{-}$; $R_3 = H$. II (IP): $R_1 = R_3 = Cl\text{-}CH_2\text{-}CH_2\text{-}$; $R_2 = H$. III (trophosphamide): $R_1 = R_2 = R_3 = Cl\text{-}CH_2\text{-}CH_2\text{-}$.

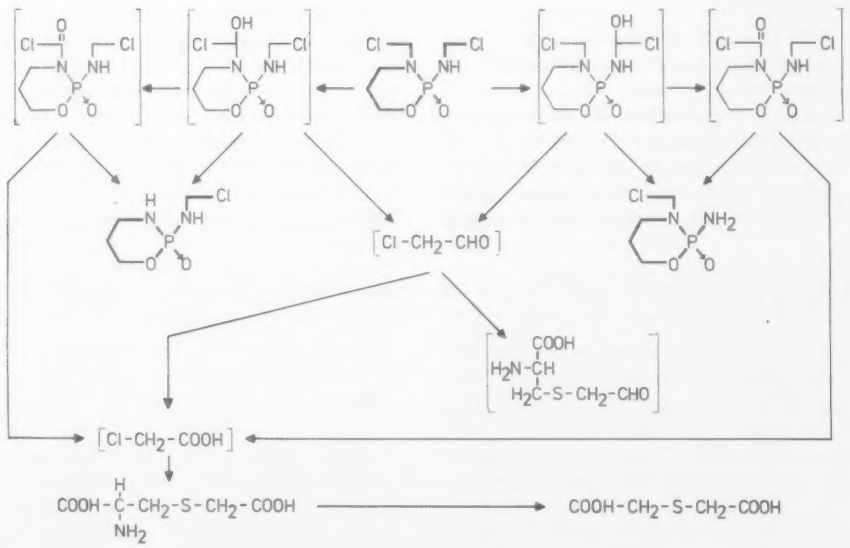


FIGURE 8.—Metabolism of IP by side-chain oxidation. The compounds in brackets were not isolated.

the metabolism of these drugs? (*c*) What is the significance of the regimen of drug application and what possibilities exist to optimize the therapeutic effect, thus establishing a more rational dosage of CP and its analogs?

REFERENCES

1. Mouridsen HT, Faber O, and Skovsted L. The biotransformation of cyclophosphamide in man: analysis of the variation in normal subjects. Acta Pharmacol Toxicol 35:98-106, 1974.
2. Norpoth K, Wüst G, and Witting U. Quantitative Bestimmung des Ifosfamids und eines Ifosfamid-Metaboliten im Patientenurin. Verh Dtsch Ges Inn Med 78:1561-1564, 1972.
3. Bakke JE, Feil VJ, Fjelstul CE, et al. Metabolism of cyclophosphamide by sheep. J Agric Food Chem 20:384-388, 1972.
4. Boughton OD, Brown RD, Bryant R, et al. Assay of cyclophosphamide. J Pharm Sci 61:97-100, 1972.
5. Brooke D, Bequette RJ, and Davis RE. Chemical stability of cyclophosphamide in parenteral solutions. Am J Hosp Pharm 30:134-137, 1973.
6. Duncan JH, Colvin OM, and Fenselau C. Mass spectrometric study of the distribution of cyclophosphamide in humans. Toxicol Appl Pharmacol 24:317-323, 1973.
7. Jackson CH, Jr. and Reynolds J. Gas-chromatographic determination of cyclophosphamide residues in sheep tissues. J Agric Food Chem 20:972-974, 1972.
8. Pantarotto C, Bossi A, Belvedere G, et al. Quantitative GLC determination of cyclophosphamide and isophosphamide in biological specimens. J Pharm Sci 63:1554-1558, 1974.
9. Allen LM, and Creaven PJ. Gas chromatographic method for the determination of plasma isophosphamide (NSC-109724). Cancer Chemother Rep 56:721-723, 1972.
10. Laux D, and Lausch RN. In vitro microassay for biological activity of cyclophosphamide metabolites. Antimicrob Agents Chemother 3:407-411, 1973.
11. Sawicki E, Bender DF, Hauser TR, et al. Five new methods for the spectrophotometric determination of alkylating agents including some extremely sensitive anticatalytic methods. Anal Chem 35:1479-1486, 1963.
12. Sawicki E, and Sawicki CR. Analysis of alkylating agents: applications to air pollution. Ann NY Acad Sci 163:895-920, 1969.
13. Jarman M. Formation of 4-ketocyclophosphamide by the oxidation of cyclophosphamide with $KMnO_4$. Experientia 29:812-814, 1973.
14. Connors TA, Cox PJ, Farmer PB, et al. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
15. Whitehouse MW, Beck FJ, and Kacena A. Some (pharmacological) properties of chloracetaldehyde, an oxidation product and potential metabolite of cyclophosphamide. Agents Actions 4:1, 1974.
16. Yllner S. Metabolism of chloroacetate-1-^{14}C in the mouse. Acta Pharmacol Toxicol 30:69-80, 1971.
17. Yllner S. Metabolism of 1,2-dichloroethane-^{14}C in the mouse. Acta Pharmacol Toxicol 30:257-265, 1971.

Studies on the Clinical Pharmacology and Toxicology of Isophosphamide (NSC-109724)[1]

Patrick J. Creaven,[2,3,*] Larry M. Allen,[2,3] Martin H. Cohen,[2] and Robert L. Nelson[2,4]

The National Cancer Institute-Veterans' Administration Medical Oncology Branch is a major center in the US for lung cancer research and our interest in isophosphamide (IP) was originally related to its potential use in lung cancer. It was based on three major factors: (*a*) reports that the activity of IP was superior to that of cyclophosphamide against certain experimental solid tumors (specifically, the Lewis lung carcinoma) (1); (*b*) reports that against human solid tumors and especially against small cell carcinoma of the lung IP was more active than the older congener (2); and (*c*) reports from preliminary trials in Germany (3) and South Africa (4) that the dose-limiting toxicity was toxicity of the urinary tract. We were particularly interested in active agents with dose-limiting toxicities other than myelosuppression for use in combinations against lung cancer. It is of interest to note that although much of the work recently done by our group with IP, as well as the work of a number of other groups, has been related to efforts to reduce the urinary-tract toxicity and make the dose-limiting toxic effect myelosuppression, one of the original bases for our interest in the drug was that its dose-limiting toxicity appeared to be other than myelosuppression.

Over the past 5 years we have conducted two initial clinical trials with IP. One was a single-dose trial (ie, a trial in which each course consisted of a single dose) which was conducted by our unit in collaboration with the Roswell Park Memorial Institute in Buffalo, NY. The other was a trial in which each course consisted of daily administration of the drug for 3 days. In the initial clinical exploration of a new drug (ie, any drug not previously used in the US), we traditionally start at one third of the lowest toxic dose level in the most sensitive animal species tested. The dose is escalated by 100% and then by decreasing increments to a level of 33% increments which is then used as the escalation factor until a toxic dose is reached. A different group of patients is entered at each dose level. As in the case of the single-dose trial, this approach frequently proves to be too cautious and is undergoing reappraisal at this time.

The study design of our single-dose trial of IP is shown in table 1. At the 5000-mg/m^2 dose, tests of tubular function were carried out on the day before and 4 days after dosage was given. Renal concentrating capacity was measured by measuring urine osmolality after a 12-hour fast, and renal tubular acidification capacity was measured by measuring the acidity of the urine after ingestion of ammonium chloride. The moderately toxic dose found in the single-dose study was 5000 mg/m^2 of body surface area, and this was used as the basis for calculating the starting dose of the daily × 3 schedule of administration. Roughly one third of this (1600 mg/m^2) was administered daily for 3 days so that the total dose given over 3 days (4800 mg/m^2) was roughly the moderately toxic dose of the agent given as a single dose. The initial escalation was by 50%, and no further escalation was necessary since the dose reached by this 50% increase (ie, 2400 mg/m^2/day × 3 days) was the moderately toxic dose. The patient eligibility for the studies was as follows:

a. Microscopically confirmed diagnosis of cancer.
b. Disease not responsive to therapy of proven value.
c. Expected survival of at least 2 months' duration.
d. At least a 2-week period since last dose of other potentially myelosuppressive therapy *and* complete recovery from reversible toxicity.
e. Minimal hematologic requirements: white blood cell (wbc) count ≥ 5000 cells/mm^3; platelet count ≥ 100,000 cells/mm^3.
f. Minimal biochemical parameters: blood urea nitrogen (BUN) level ≤ 25 mg/100 ml; serum creatinine level ≤ 1.5 mg/100 ml; serum bilirubin level < 1.5 mg/100 ml; serum glutamic oxaloacetic transaminase (SGOT) level < 100 international units.

---

[1]Isophosphamide: CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide.

[2]National Cancer Institute-Veterans' Administration (VA) Medical Oncology Branch, VA Hospital, Washington, DC.

[3]*Present address:* Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY.

[4]*Present address:* Lilly Research Laboratories, Clinical Pharmacology Unit, Marion County General Hospital, Indianapolis, Ind.

**Reprint requests to:* Patrick J. Creaven, MD, Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

TABLE 1.—Study design: single high-dose IP*

Drug administration	Iv, in 500 ml over 30 mins
Starting dose	200 mg/m²
Escalation	Modified Fibonacci search
No. of patients	3 at nontoxic dose levels; 6 or more at toxic dose levels
Hydration	2000 ml/m²/day from Day -1 to Days 2 or 3

* At 5000 mg/m² patients received 2 doses at an interval of 3 wks. During the first or second treatment (determined by randomization) patients received bladder irrigation with 1% *N*-acetyl-L-cysteine in 2000 ml of saline/day.

g. Written informed consent.
h. Absence of acute intercurrent complications or pregnancy.

The periodic observations are shown in table 2. The toxic effects seen with a single dose of IP are shown in table 3 (5). The largest dose used was 10,000 mg/m² of body surface area which proved to be too toxic and resulted in two early deaths. At the moderately toxic dose (5000 mg/m²) six of ten patients had leukopenia with wbc counts < 4000 cells/mm³. However, the predominant dose-limiting toxicity was that of the urinary tract. Nausea and vomiting was universal in all patients treated at doses of ≥ 3800 mg/m² and a considerable amount of toxicity of the central nervous system was seen at the higher doses. This consisted mainly of confusion and lethargy occurring within the first 24 hours after treatment.

The daily × 3 schedule was designed to attempt to reduce the urinary-tract toxicity and enable more drug to be given. Although, as will be discussed in detail later, bladder washout with *N*-acetyl-L-cysteine was capable of obviating bladder toxicity from the high-single dose, this made administration of the drug very cumbersome and it was essentially limited to use in patients who were hospitalized. It was hoped that a daily × 3 administration would reduce the urinary-tract toxicity and at the same time enable the drug to be given on an outpatient basis. The toxicity seen at the two doses investigated is shown in table 4 (6). Eight patients were treated at 1600 mg/m²/day and 12 at 2400 mg/m²/day. This second dose was considered to be the maximum tolerated dose and we did not explore doses greater than this. One patient developed acute tubular necrosis and subsequently died on this dose (table 4). Although there were other factors involved in this case, it is likely that drug toxicity at least contributed to the patient's death. Although we were able to give a larger total amount of drug (gross hematuria was not seen at

TABLE 2.—IP clinical study (observations and frequency)

Parameter	Frequency of observation: Twice weekly	Weekly
Body weight		×
Wbc and platelets	×	
Hemoglobin or hematocrit		×
Differential	×	
Reticulocytes	×	
Bilirubin		×
Alkaline phosphatase		×
Lactic dehydrogenase		×
SGOT or serum glutamic pyruvic transaminase		×
BUN		×
Serum creatinine		×
Creatinine clearance*		×
Uric acid		×
Urinalysis (daily, Days 1-4)		×

*Daily × 3 study only.

TABLE 3.—IP toxicity: single dose

	Dose (mg/m²)				
	200-2900	3800	5000	7000	10,000
No. of patients entered	28	6	10	4	3
Leukopenia (wbc count < 4000 cells/mm³)	1	3	6	3	1*
Thrombocytopenia (platelet count < 10^5 cells/mm³)	0	0	0	0	0
Nausea and vomiting	5	6	10	4	3
Lethargy/confusion	0	0	4	1	2
Urinary-tract toxicity	3	4	7	2	2

*There were 2 early deaths at this dose.

TABLE 4.—IP toxicity: daily × 3 schedule

	Dose (mg/m^2/day)	
	1600	2400
No. of patients entered	8	12
Leukopenia (wbc count < 4000 cells/mm^3)	4	6
Thrombocytopenia (platelet count < 10^5 cells/mm^3)	1	0
Nausea and vomiting	5	11
Lethargy/confusion	2	6
Alopecia (No. of evaluable cases)	3(4)	5(7)
Oliguria	2	3
Microscopic hematuria (alone)	7	5
Gross hematuria	0	4
Acute tubular necrosis	0	1

1600 mg/m^2/day) we were disappointed to find that the dose-limiting toxicity on this schedule was still urinary-tract toxicity in the majority of cases. The pattern of toxicity is in fact very similar to the pattern seen with the high-single dose.

Tables 5 and 6 give a more detailed comparison of the two dose schedules with regard to hematuria and leukopenia. Hematuria was considerably more noticeable with 5000 mg/m^2 as a single dose than with 1600 mg/m^2/day for 3 days. In terms of leukopenia, both daily × 3 doses seem roughly comparable with 5000 mg/m^2 given as a single dose.

The urinary-tract toxicity of IP in both the single dose and in the daily × 3 schedule led us to conduct a number of pharmacologic investigations to try to identify the determinants of toxicity which might be susceptible to manipulation. As anticipated, the toxicity is related to the excretion of alkylating activity in the urine. Thus, the amount of alkylating activity in the urine after a moderately toxic single dose of IP (5000 mg/m^2) is higher at each 8-hour period for the first 3 days after dosage than for a moderately toxic dose of cyclophosphamide (1100 mg/m^2) (fig 1), the dose-limiting toxicity of which is usually myelosuppression. Irrigation of the bladder for 24 hours after a 5000-mg/m^2 dose of IP with a 1% solution of *N*-acetyl-L-cysteine (2 liters/day) was totally effective in abolishing bladder toxicity of the drug. However, *N*-acetyl-L-cysteine appears not to affect the amount of alkylating activity in the urine (table 7). The reason for this can be seen in table 8 where urine

TABLE 5.— Hematuria after IP

Dose (mg/m^2)	No. of patients—			Mean duration (days)*
	Treated	With hematuria		
		Microscopic	Macroscopic	
3800	6	1	3	3
5000	10	3	4	4
1600 daily × 3	8	7	0	2
2400 daily × 3	12	5	4	2

*Rounded to the nearest whole No.

TABLE 6.—Leukopenia after IP

Dose (mg/m^2)	No. of patients—		Mean wbc nadir (× $10^{-3}/mm^3$)	Mean No. of days to nadir*	Day of recovery to wbc count of > 4000 cells/mm^3
	Treated	With leukopenia			
3800	6	3	3.1	8	11
5000	10	6	3.0	8	12
7000	4	3	1.9	9	20
1600 daily × 3	8	4	2.9	11	15
2400 daily × 3	12	6	2.9	11	15

*Rounded to the nearest whole No.

TABLE 7.—Alkylating activity excreted* (μmol of mechlorethamine equivalent)

Patient No.	No irrigation	Bladder irrigated with 1% *N*-acetyl-L-cysteine
1	2372.4	2332.0
2	1889.6	1942.7

* Dose = 5000 mg/m² of IP.

TABLE 8.—Effect of *N*-acetyl-L-cysteine on urinary alkylating activity

	Total alkylating activity after a 30-min incubation with 1% *N*-acetyl-L-cysteine (% of initial)	
	24°C	37°C
Urine from a patient who received IP	99.26%	95.96%
Normal urine to which mechlorethamine was added before incubation	82.89%	39.80%

from a patient who had received IP was incubated with 1% *N*-acetyl-L-cysteine in vitro for 30 minutes both at 24°C and at 37°C with no appreciable effect on the total alkylating activity even though in a control experiment with mechlorethamine there was a marked effect at 37°C and a significant effect even at 24°C (7). We therefore tentatively concluded that the effect of the bladder washout might be largely a dilutional one and this is in line with our observations that the factors most clearly related to bladder toxicity are urine volume and concentration of alkylating activity. However, this conclusion must now be modified in light of data presented at this meeting (8). It is difficult to make good correlations with the small number of patients investigated, particularly with the large individual variations in toxicity, but we have attempted to make such a correlation (table 9). For

TABLE 9.—IP urinary-tract toxicity (5000 mg/m²)

Patient No.	Alkylating activity concentration in urine* (nmols/ml)	Urinary-tract toxicity score†
1	1083	21
2	1153	20
3	1108	15
4	813	2
5	546	0
6	548	0
7	490	0

*Mean of the 2 highest 8-hr concentrations.

†Score = severity × No. of days. Severity: 1 = frequency; 2 = dysuria; 3 = hematuria.

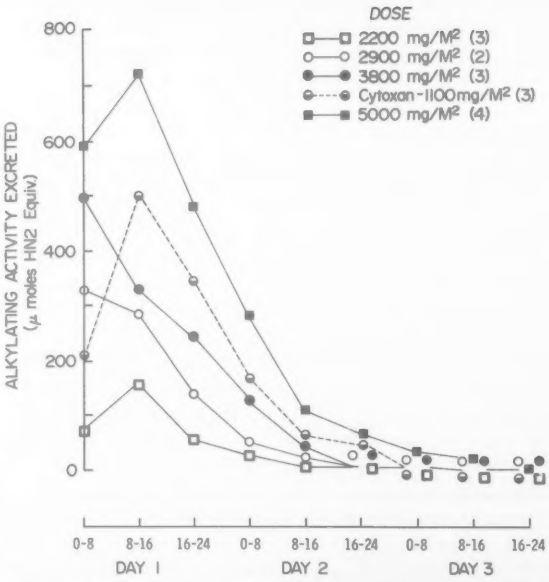


FIGURE 1.—Total excretion of alkylating activity for each 8-hr period during the 72 hrs after infusion of 2200-5000 mg/m² of IP (solid lines) and 1100 mg/m² of cyclophosphamide (interrupted line). Each point represents the mean from the No. of patients given in parentheses after the dose.

the purposes of this and the next table, we have quantitated urinary-tract toxicity by multiplying the severity on a scale of 1-3 (1 = frequency with no dysuria, 2 = frequency and dysuria, and 3 = hematuria) by the number of days that these symptoms were experienced. This is clearly a rather crude measure but it enables one to have a single number to represent urinary-tract toxicity. If the seven patients we investigated at 5000 mg/m² are arranged in order of their urinary-tract toxicity scores, this is roughly the order of the mean of the two highest 8-hour concentrations of alkylating activity in the urine expressed in terms of nmols/ml of mechlorethamine equivalent. Other attempts to correlate toxicity with pharmacologic parameters are again limited by the small number of patients involved, but if we look at the area under the curve (AUC) of the plasma alkylating activity (table 10), it is clear that in the case of the five patients investigated the overall total toxicity is in the same order. However, the fact that most of the numbers are roughly the same, except for one patient who had a very large AUC, minimizes the value of this observation. It is of interest that in this small series, patients with the shortest half-life of unchanged IP have the most toxicity and the patients with the longest half-life have the least. This might be expected if one considers that

TABLE 10.—IP toxicity and pharmacokinetic parameters (5000 mg/m²)

Patient No.	AUC of plasma alkylating activity (nmols × hr/ml)	β-phase half-life of unchanged drug in plasma (hrs)	Genitourinary toxicity score*	Wbc depression†
3	1071	10.34	15	4.6
2	558	13.59	20	1.3
1	516	—	21	0
4	503	14.74	2	1.0
5	407	19.80	0	0

*Score = severity × No. of days. Severity: 1 = frequency; 2 = dysuria; 3 = hematuria.

†5000 − nadir × 10^{-3}.

it is the metabolite which is causing the toxicity. Total mean urinary alkylating activity after a dose of 1600 mg/m²/day × 3 was a little lower (1657 μmols of mechlorethamine equivalent) than the mean after a dose of 5000 mg/m² as a single dose (2189 μmols of mechlorethamine equivalent) but the difference was not significant. Again we are inclined to believe that the lower toxicity with this schedule is due to the fact that the alkylating activity is excreted over a longer period of time and that the same high concentrations achieved with the single dose are never reached.

Our conclusions from these studies are (*a*) that the dose-limiting toxicity with IP is nearly always urinary-tract toxicity as has been found by others; (*b*) that this can be obviated by the use of bladder irrigation, the effect of which is probably partly dilutional; (*c*) that it is possible to give a larger dose of IP on a 3-day schedule than on a single-dose schedule, although the nature of the dose-limiting toxicity is not altered; and (*d*) that the factors which correlate best with toxicity are the concentration of urinary alkylating activity, urine volume, and the AUC of total alkylating activity. There is a suggestion from the data that a shorter half-life of unchanged drug may lead to a greater toxicity.

REFERENCES

1. BROCK N. Pharmacologische untersuchungen mit neuen *N*-chloroathyl-phosphoraureester-diamiden. *In* Proceedings of the 5th International Congress of Chemotherapy (Spitzy KH, and Haskek H, eds). Vienna, Verlag der Wiener Medizinischen Akademie, 1967, vol 2, p 1155.
2. SCHEEF W. Problems, experience, and results of clinical investigations with ifosfamide. *In* Advances in Antimicrobial and Antineoplastic Chemotherapy (Proceedings of the 7th International Congress of Chemotherapy, Prague, 1971) (Hejzlar M, Semonský M, and Masák S, eds). Baltimore, University Park Press, 1972, vol 2, pp 797-800.
3. BROCK N, HOEFER-JANKER H, HOHORST HJ, ET AL. Die aktivierung von ifosfamid an mensch und tier. Arzneim Forsch 23:1-14, 1973.
4. VAN DYK JJ, FALKSON HC, VAN DER MERWE AM, ET AL. Unexpected toxicity in patients treated with iphosphamide. Cancer Res 32:921-924, 1972.
5. COHEN MH, CREAVEN PJ, TEJADA F, ET AL. Phase I clinical trial of isophosphamide (NSC-109724). Cancer Chemother Rep 59:751-763, 1975.
6. NELSON RL, CREAVEN PJ, COHEN MH, ET AL. Phase I clinical trial of a 3 day divided dose schedule of ifosfamide (NSC-109724). Eur J Cancer, 1976. In press.
7. CREAVEN PJ, ALLEN LM, ALFORD DA, ET AL. Clinical pharmacology of isophosphamide. Clin Pharmacol Ther 16:77-86, 1974.
8. DRAEGER U, PETER G, and HOHORST H-J. Deactivation of cyclophosphamide (NSC-26271) metabolites by sulfhydryl compounds. Cancer Treat Rep 60:355-359, 1976.

Studies on the Human Pharmacokinetics of Isophosphamide (NSC-109724)[1]

Larry M. Allen,[2,3,*] Patrick J. Creaven,[2,3] and Robert L. Nelson[2,4]

SUMMARY

The pharmacokinetics of isophosphamide (IP) in man has been studied using ^{14}C-labeled drug and differential extraction of unchanged drug from metabolites by $CHCl_3$ extraction of plasma and urine. The plasma decay of IP is biphasic with a terminal half-life of 15.2 hours which is twice that reported for cyclophosphamide (CP). The fraction of drug metabolized is 49% for IP compared to 88% reported for CP. This is consistent with the six-times larger pseudometabolic pharmacokinetic rate constant reported for CP (0.26 hr^{-1}) than found by us for IP (0.04 hr^{-1}). The renal clearance for IP (21.3 ml/min) is two times that of CP (10.7 ml/min). In addition to an analysis of the kinetics of disposition and elimination of unchanged IP, an analysis of the pharmacokinetics of total metabolite of IP in plasma and urine was made. A nonlinear pharmacokinetic model which includes a term for biotransformation according to Michaelis and Menten revealed that the total metabolites do not diffuse from a small plasma space of 2.1 liters but are excreted directly via the urine. In contrast to the biexponential plasma decay of single, high-dose IP (5 g/m^2), multiple-dose IP (2.4 g/m^2/day × 3) shows a monoexponential decay with a half-life of 6.9 hours, a pseudometabolic rate constant of 0.08 $hour^{-1}$, and a renal clearance of 18.7 ml/minute. The fraction of drug metabolized (79.7%) calculated from the pharmacokinetic parameters agrees with the fraction of drug recovered in urine as total metabolites (72.8%). This is distinctly different from high-dose IP (48.6%) but similar to the fraction of drug metabolized as reported for CP (88.0%). This suggests that the pharmacokinetic transfer constant for IP metabolism is dose dependent, an observation that may be useful in developing new IP regimens.

[Cancer Treat Rep 60:451-458, 1976]

A significant therapeutic determinant in the chemotherapy of cancer is the kinetics of drug disposition and elimination. This is particularly true for the pro-drugs cyclophosphamide (CP) and isophosphamide (IP) which require hepatic biotransformation for generation of cytostatic products. Other things being equal, the optimum chemotherapeutic benefit between drugs will depend on a balance between the rates of drug distribution, biotransformation, and excretion.

We have shown (fig 1) with in vitro rat liver microsomal preparations that the kinetics of CP and IP activation differ only in the concentration required for half maximal velocity (K_m), where the concentration of IP required to produce the same rate of activation is five times that of CP (1). We have suggested that the reason for this difference is the bulky chloroethyl group on the endocyclic nitrogen of IP (2). If we assume that the site of the initial enzymatic attack on IP is the same as for CP (fig 2), ie, the carbon adjacent to the endocyclic nitrogen, it can be seen in figure 3 that this site is sterically hindered by the presence of a chloroethyl side chain which may reduce the approach of this carbon to the active site of the enzyme. Furthermore, this activation of IP was obligatory for

[1]Isophosphamide: CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide.

[2]National Cancer Institute-Veterans' Administration (VA) Medical Oncology Branch, VA Hospital, Washington, DC.

[3]*Present address:* Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY.

[4]*Present address:* Lilly Research Laboratories, Clinical Pharmacology Unit, Marion County General Hospital, Indianapolis, Ind.

**Reprint requests to:* Larry M. Allen, PhD, Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

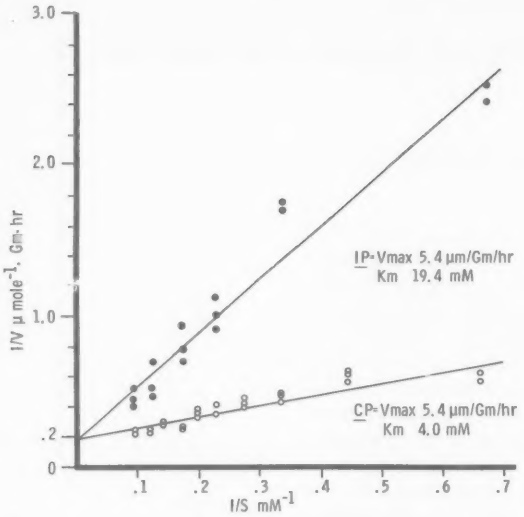


FIGURE 1.—Lineweaver-Burke plot of the activation of IP and CP as described in ref 1.

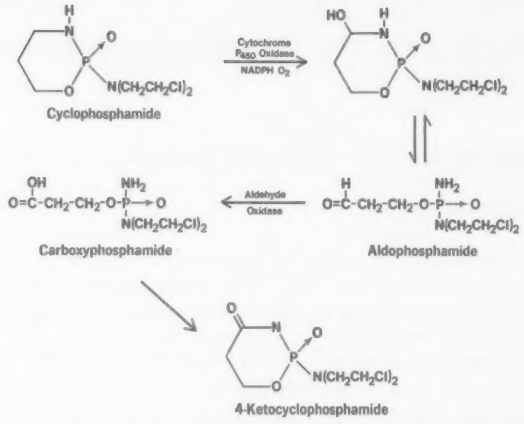
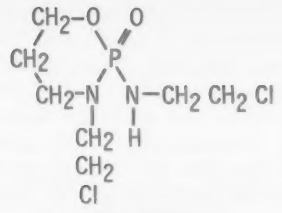


FIGURE 2.—Proposed route of CP metabolism (*from* ref 2).

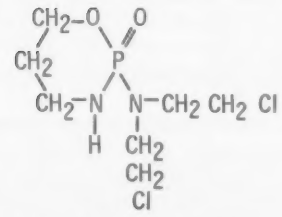
TABLE 1.—Coefficients of biexponential decay function and β-phase half-life for unchanged IP in plasma after short iv infusion*

Constant	Serum concentration (±SE) in patient No.— 1	2	3	4	5
A, μg/ml	34.99 (7.60)	66.24 (16.69)	50.00 (20.53)	118.31 (10.93)	149.97 (7.00)
α, hr^{-1}	0.65 (0.16)	0.60 (0.34)	0.60 (0.29)	0.49 (0.12)	0.29 (0.02)
B, μg/ml	124.47 (4.04)	145.50 (17.75)	192.60 (11.17)	146.02 (11.05)	100.40 (7.04)
β, hr^{-1}	0.040 (0.003)	0.067 (0.010)	0.047 (0.004)	0.051 (0.005)	0.035 (0.003)
β-phase half-life, hr	17.33	10.34	14.74	13.59	19.80

*Distribution and elimination constants for unchanged IP in serum of 5 patients determined from the biexponential decay function $Ae^{-\alpha t} + Be^{-\beta t}$.



IFOSFAMIDE



CYCLOPHOSPHAMIDE

FIGURE 3.—Chemical structures of IP and CP.

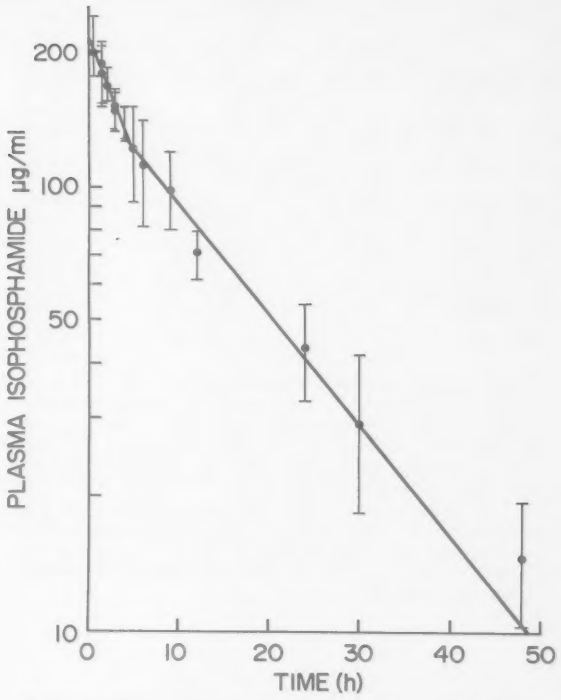


FIGURE 4.—Plasma decay of IP in patients receiving 5 g/m². Each point represents a mean from 5 patients ± SD (*from* ref 8).

TABLE 2.—Comparison of some selected pharmacokinetic parameters for CP and IP

Parameters	CP* (mean ± SD)	IP (mean ± SD)
Distribution		
V_c, † liters	21.6 ± 6.3	34.0 ± 2.5
K_{12}, ‡ hr^{-1}	2.9 ± 1.5	0.14 ± 0.04
K_{21}, ‡ hr^{-1}	4.2 ± 2.5	0.37 ± 0.16
Elimination		
K_{el}, § hr^{-1}	0.29 ± 0.11	0.07 ± 0.02
k_m, ¶ hr^{-1}	0.26 ± 0.10	0.04 ± 0.01
Cl, ** ml/min	10.7 ± 3.3	21.3 ± 5.9

*From ref 6.

†Central compartment volume for 2-compartment model.

‡Transfer constant for drug transport from compartment 1 (plasma equivalent space) to peripheral compartment 2 and vice versa respectively.

§Transfer constant for elimination (all processes).

¶This is a hybrid metabolic rate constant as used by Cohen et al (6) and is to be distinguished from K_m, the Michaelis constant, as used elsewhere; k_m is derived from the Michaelis-Menten equation where K_m is assumed to be >> substrate concentration, therefore, $k_m = V_{max}/K_m$. The k_m derived from our nonlinear model is 0.034 (± 0.013).

**Renal clearance.

protein and DNA binding and cytotoxic activity against L1210 leukemia cells in culture (3,4).

During our phase I clinical trial of IP we studied the clinical pharmacology and pharmacokinetics with the aim of comparing the results with those available for CP in man (5-7).

IP labeled with ^{14}C in the side chain was given as a single short iv infusion. Measurements were made of unchanged drug and total radioactive metabolites in the plasma and urine by differential extraction with $CHCl_3$ (8). The alkylating activity in the plasma and urine was measured by a modification of the nitrobenzyl pyridine method of Friedman and Boger (9). The decay of unchanged IP in plasma best fitted a biexponential equation (fig 4); some of the parameters obtained from this fit are given in table 1 (10,11). The β phase or terminal half-life is two to three times greater for IP than for CP (5-7). The four parameters (A, α, B, and β) which characterize the plasma-decay function were used to calculate the transfer constants for a two-compartment open pharmacokinetic model (12,13). A comparison of the transfer constants for IP and those reported for CP (6) are shown in table 2. A

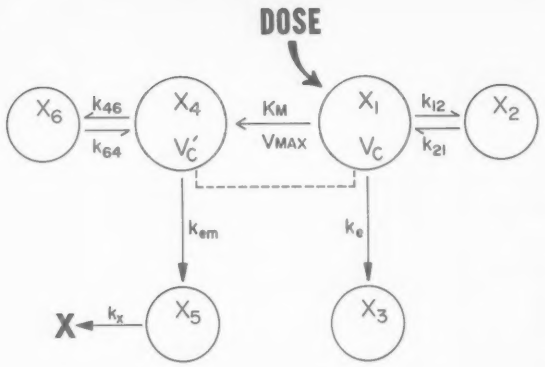


FIGURE 5.—Multicompartment pharmacokinetic model. X_1 and X_4, central compartments; X_3 and X_6, urine; X_2 and X_5, peripheral compartments for IP and metabolite(s) respectively. V_{max} and K_m, constants for metabolism according to Michaelis and Menten; k_x, transfer rate constant (hr^{-1}) for irreversible biologic alkylation or drug decomposition; k_{ij} (i = 1,2,3. . .n, j = 1,2, 3. . .m), transfer rate constants (hr^{-1}) for drug transport into compartment j from compartment i; K_{em}, transfer constant for urinary excretion of metabolite(s); V_c and V_c', volumes of the central compartment for IP and metabolite(s) respectively (*from* refs 10 and 11).

$$\frac{dX_1}{dT} = k_{21} \cdot X_2 - (k_e + k_{12}) \cdot X_1 - \frac{V_M \cdot X_1}{K_M + X_1}$$

$$\frac{dX_2}{dT} = k_{12} \cdot X_1 - k_{21} \cdot X_2$$

$$\frac{dX_3}{dT} = k_e \cdot X_1$$

$$\frac{dX_4}{dT} = \frac{V_M \cdot X_1}{K_M + X_1} - (k_{em} + k_{45}) \cdot X_4 + k_{54} \cdot X_5$$

$$\frac{dX_5}{dT} = k_{45} \cdot X_4 - k_{54} \cdot X_5$$

$$\frac{dX_6}{dT} = k_{em} \cdot X_4 - k_x \cdot X_6$$

FIGURE 6.—Differential equations used for model shown in fig 5 where K_m is the Michaelis-Menten constant (*from* ref 11).

number of the model parameters including the volumes of the central compartment, the elimination rate constant, and the pseudometabolic rate constant are markedly different for the two drugs. The magnitude of the difference between the pseudometabolic pharmacokinetic rate constants of CP and IP is in the same direction as we found in vitro for the Michaelis constant of rat liver hepatic microsomal activation. Therefore, we built a multicompartment nonlinear pharmacokinetic model for IP with a term to account for biotransformation according to Michaelis and Menten (14). The generalized model is shown in figure 5 and its mathematic description using differential equations is outlined in figure 6.

The first three equations were fitted simultaneously to unchanged IP in plasma and urine expressed as a percent of dose. The transfer constants for these three equations were then held constant and the data for total metabolite in the plasma and urine were fitted to the remaining three equations. In order to improve the fit of the model to the data it was necessary to introduce an irreversible transfer factor (k_x) representing the loss of radioactivity either from its conversion to a volatile product or alkylation of the renal tract. The volume of distribution of the total metabolites in plasma was obtained by parametric adjustment during computer-aided fittings of equations in figure 6. We found the transfer constants for metabolite distribution into peripheral space to be negligible and on the order of 0.001 $hour^{-1}$. The kinetic parameters derived from fitting the model to the data are shown in table 3. The excretion rate constant for unchanged drug is small and relatively invariant; that for metabolite is larger but quite variable. There was also a large degree of variation between patients in the derived Michaelis constant for metabolism. Some further characteristics of the model for unchanged drug are shown in table 4. The large central compartment volume for unchanged IP represents plasma and easily perfused tissue space whereas the central compartment volume for the metabolites was found to be 2.8-7.5 liters and indicates limited diffusion from plasma in accordance with our previous findings on DNA and protein binding of in vitro activated IP (3,4). As shown in table 4 we found excellent agreement between the percent of metabolite recovered in the urine and that predicted by the model. The agreement between the data and the model for two patients is shown in figures 7A and 7B for unchanged drug and metabolite. In addition, we found that the concentration of IP in the cerebrospinal fluid (CSF) in two patients closely corresponded with the concentration of IP in the peripheral

TABLE 3.—Summary of pharmacokinetic parameters for a multicompartmental nonlinear model for IP in man*

Parameters	Patient No. and dose (mg)				
	1(5320)	2(6750)	3(7750)	4(9200)	5(9500)
K_{12},† hr^{-1}	0.100 (0.010)	0.166 (0.040)	0.150 (0.024)	0.175 (0.12)	0.064 (0.008)
K_{21},† hr^{-1}	0.306 (0.053)	0.297 (0.104)	0.655 (0.142)	0.353 (0.041)	0.120 (0.035)
K_e,‡ hr^{-1}	0.024 (0.001)	0.041 (0.003)	0.034 (0.001)	0.037 (0.001)	0.064 (0.002)
V_{max},§ mg/ml/hr	0.022 (0.002)	0.041 (0.002)	0.017 (0.002)	0.008 (0.001)	0.024 (0.003)
K_m,§ mg/ml	1.040 (0.005)	0.965 (0.003)	0.711 (0.007)	0.151 (0.005)	0.808 (0.010)
K_{em},¶ hr^{-1}	8.92 (0.42)	0.662 (0.249)	0.134 (0.087)	18.27 (0.31)	3.62 (0.59)
K_x,** hr^{-1}	0.007 (0.001)	0.007 (0.001)	0.008 (0.005)	0.006 (0.002)	0.006 (0.002)

*All values are followed by ± SE in parentheses.
†See table 2 for definitions.
‡Excretion rate constant for urinary elimination.
§Constants for metabolism according to Michaelis and Menten.
¶Excretion rate constant for metabolite(s) urinary elimination.
**Transfer constant for irreversible biologic alkylation or drug decomposition in the urine or urinary tract (see fig 5).

TABLE 4.—Summary of model compartment volumes, urine recovery of unchanged drug and its metabolites, and renal clearance for IP

Parameters	Patient No. and body weight (kg)				
	1 (50)	2 (48)	3 (44)	4 (74)	5 (77)
Compartment volume (liters)					
Central (V_c)	33.4	31.9	32.0	34.8	37.9
Peripheral	10.9	17.9	7.3	17.3	20.2
Urine excretion, 72 hrs (%)*					
IP (found)	47.7	47.7	57.1	41.1	66.0
	(49.3)	(48.1)	(60.9)	(41.9)	(65.2)
Metabolites (found)	29.8	36.3	23.5	26.8	20.5
	(30.1)	(33.5)	(24.0)	(27.8)	(20.1)
Renal clearance, Cl (ml/min)†	8.3	12.8	13.3	19.0	29.1

*Nos. in parentheses are percentages actually recovered; those preceding parentheses are percentages predicted by the model.
†This was determined experimentally from the slope of the line for the excretion rate plotted against the actual or interpolated plasma concentration at the midpoints of the urine excretion interval. The theoretical renal clearances determined by $k_e \times V_c$ were 13.4, 21.8, 18.1, 21.4, and 40.4 ml/min respectively.

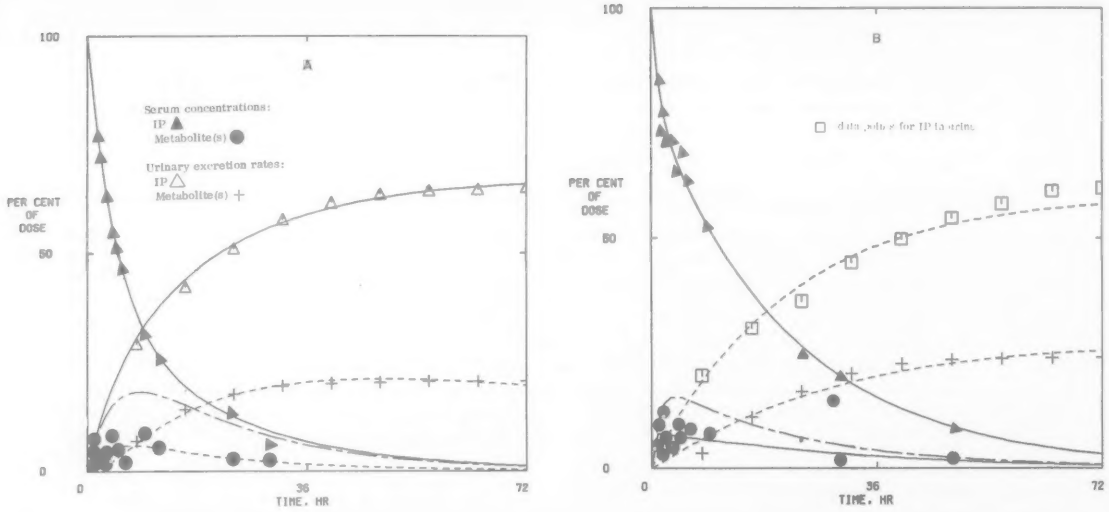


FIGURE 7.—Plot of percent of dose of IP or its metabolite(s) in the respective compartments of the pharmacokinetic model (*from* refs 10 and 11). A. Data points for patient No. 5 (9500 mg) were obtained experimentally from serum concentrations of IP and its metabolite(s), and from urinary excretion rates of IP and its metabolite(s). (– – – –) = theoretical levels of the drug in peripheral compartment X_2 of the model (*see* fig 5). B. Same as A but for patient No. 3 (7750 mg).

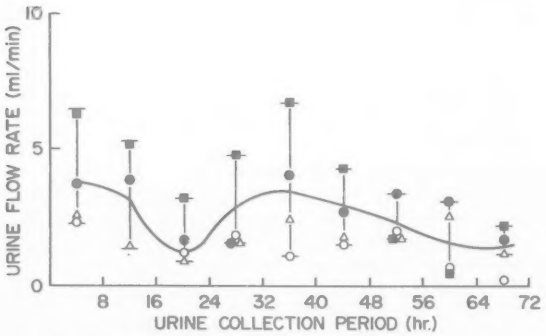


FIGURE 8.—Urine flow rate after IP at 5 g/m². Solid line is the result of a 5-point hyperbolic smoothing function applied to all the points.

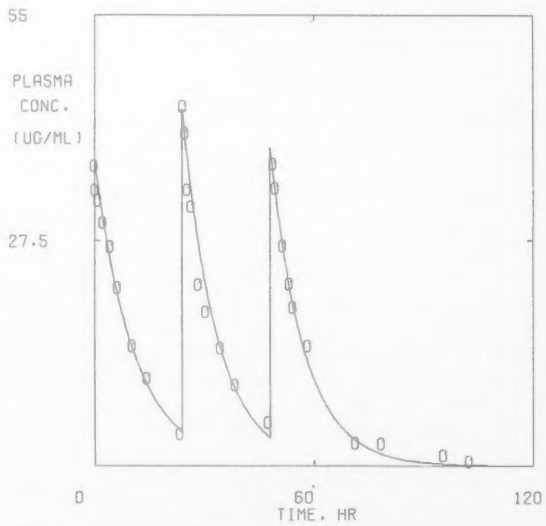


FIGURE 9.—Plasma levels of IP for patient No. 7 after multiple-dose administration (1600 mg/m²). Symbols are data and the solid line is the result of computer-aided fit to data according to a 1-compartment open pharmacokinetic model.

space as predicted from the model. The IP concentration in the CSF was 49.4 μg/ml at 3 hours after infusion for patient No. 2 and 52.9 μg/ml at 2 hours after infusion for patient No. 5. These concentrations would correspond to a volume of distribution of 12.1 and 18.6 liters respectively. The corresponding model-predicted volumes are 17.8 and 19.6 liters.

In all patients who were studied the urine-flow rate decreased slightly after administration of IP (fig 8). This phenomenon may explain the low renal clearance of IP in the absence of any renal tubular reabsorption of the drug. A similar effect has been previously reported for CP (15). It was conjectured that the antidiuresis was due to specific interaction of CP at the renal sites and not due to its metabolites because mechlorethamine did not have this effect.

TABLE 5.—Computer-estimated post-infusion parameters and statistics for unchanged drug fit to monoexponential decay function for multiple-dose regimen of IP given daily for 3 days

Patient No. and body weight (kg)	Dose	Day	Constant* A, mg/liter	Constant* α, hr^{-1}
6 (58)	2480 mg (1600 mg/m^2)	1	42.8 (3.9)	0.087 (0.007)
		2	35.6 (3.3)	0.124 (0.009)
		3	24.0 (5.0)	0.113 (0.014)
7(49)	2240 mg (1600 mg/m^2)	1	42.8 (0.9)	0.088 (0.002)
		2	38.3 (1.5)	0.103 (0.004)
		3	34.9 (2.7)	0.110 (0.004)
8 (57)	3600 mg (2400 mg/m^2)	1	58.8 (2.1)	0.079 (0.003)
		2	58.7 (2.3)	0.127 (0.002)
		3	38.6 (5.4)	0.104 (0.009)

*All values are followed by ± SE in parentheses.

TABLE 6.—Comparison of pharmacokinetic parameters for single-dose and divided-dose IP

Parameters	Single-dose IP* (3.8-5 g/m^2)	Divided-dose IP* (1.6-2.4 g/m^2/day × 3 days)
Plasma decay	Biexponential	Monoexponential
Model	2-compartment open	1-compartment open
k_{el},† hr^{-1}	0.07 ± 0.02	0.104 ± 0.002
$t_{½}$,‡ hr	15.2 ± 3.6 (β-phase)	6.9 ± 0.1
V_{dss}§ (% body weight)	85.5 ± 13.1	124.7 ± 2.9
Renal clearance, ml/min	21.3 ± 5.9	18.7 ± 2.0
k_e,¶ hr^{-1}	0.03 ± 0.01	0.021 ± 0.002
k_m,¶ hr^{-1}	0.04 ± 0.01	0.083 ± 0.001
F_m**	0.486 ± 0.031	0.799 ± 0.017
Urine recovery (%)		
Total	80.2 ± 6.3	56.6 ± 4.7
Metabolite	27.1 ± 5.2	41.2 ± 2.8
Unchanged	53.1 ± 9.6	15.4 ± 2.0

*Mean ± SE.
†Elimination rate.
‡Plasma-decay half-life.
§Volume of distribution at steady state.
¶See table 2 (k_m) or table 3 (k_e) for definitions.
**Fraction of drug metabolized.

The plasma level of unchanged IP follows a biexponential decay after a single dose of 3.8-5 g/m^2 whereas the plasma level after small doses of 1.6-2.4 g/m^2/day × 3 days conforms best[5] to a monoexponential decay function (fig 9 and table 5). Furthermore, the half-life of plasma decay is only 6.9 hours compared to 15 hours for the β phase following high-dose IP and is more nearly equivalent to the half-life of the β phase reported for CP (6.5 hrs). Table 6 is a comparison of the pharmacokinetic parameters between high- and low-dose IP. The difference which is likely to be the most significant clinically is the large difference in fraction of drug metabolized. IP given in divided doses is metabolized to the extent of 80% whereas a single high dose is metabolized to an extent of only 49%. The fraction of CP metabolized in man has been reported to be 88% (8). Therefore, the transfer constant for metabolism is apparently dose dependent which is compatible with a saturable biotransformation process and with the idea that a single high dose of IP may be inhibitory to its own metabolism.

The contrasts in the pharmacokinetics of IP disposition and elimination (between single high and divided doses) may be useful in designing multiple-dose regimens of the drug.

[5]Nelson RL, Creaven PJ, Cohen MH, et al. Phase I clinical trial of a 3 day divided dose schedule of ifosfamide (NSC 109724). Eur J Cancer, 1976. In press.

REFERENCES

1. Allen LM, and Creaven PJ. In vitro activation of isophosphamide (NSC-109724), a new oxazaphosphorine, by rat liver microsomes. Cancer Chemother Rep 56:603-610, 1972.
2. Struck RF, Kirk MC, Mellett LB, et al. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
3. Allen LM, and Creaven PJ. Effect of microsomal activation on interaction between isophosphamide and DNA. J Pharm Sci 61:2009-2011, 1972.
4. ———. Interaction of mechlorethamine and isophosphamide with bovine serum albumin and rat liver microsomes. J Pharm Sci 62:854-856, 1973.
5. Bagley CM, Jr, Bostick FW, and DeVita VT, Jr. Clinical pharmacology of cyclophosphamide. Cancer Res 33:226-233, 1973.
6. Cohen JL, Jao JY, and Jusko WJ. Pharmacokinetics of cyclophosphamide in man. Br J Pharmacol 43:677-680, 1971.
7. Mellett LB, el-Dareer SM, Luce JK, et al. Activation of cyclophosphamide in various species. Pharmacologist 11:273, 1969.
8. Creaven PJ, Allen LM, Alford DA, et al. Clinical pharmacology of isophosphamide. Clin Pharmacol Ther 16:77-86, 1974.
9. Friedman OM, and Boger E. Colorimetric estimation of nitrogen mustards in aqueous media. Hydrolytic behavior of bis-(2-chloroethyl)-amine, *nor*-NH_2. Anal Chem 33:906-910, 1961.
10. Allen LM, and Creaven PJ. Pharmacokinetics of ifosfamide. Clin Pharmacol Ther 17:492-498, 1975.
11. ———. Human pharmacokinetic model for isophosphamide (NSC-109724). Cancer Chemother Rep 59:877-882, 1975.
12. Wagner JG. Biopharmaceutics and Relevant Pharmacokinetics. Hamilton, Ill, Drug Intelligence Publications, 1971.
13. Jusko WJ, and Gibaldi M. Effects of change in elimination on various parameters of the two-compartment open model. J Pharm Sci 61:1270-1273, 1972.
14. Kruger-Theimer E, and Levine RR. The solution of pharmacological problems with computers. 8. Non first-order models of drug metabolism. Arzneim Forsch 18:1575-1579, 1968.
15. Steele TH, Serpick AA, and Block JB. Antidiuretic response to cyclophosphamide in man. J Pharmacol Exp Ther 185:245-253, 1973.

Some Studies on the Distribution and Effects of Cyclophosphamide (NSC-26271) in Normal and Neoplastic Tissues[1]

Peter J. Houghton,* Kenneth D. Tew, and David M. Taylor[2]

SUMMARY

The gross uptake and intracellular distribution of ^{32}P-cyclophosphamide has been studied in normal and neoplastic rat and mouse tissues. Prolonged alkylation of DNA and RNA occurs over periods of 24 hours in the mouse and 48 hours in the rat, and it is suggested that this may involve slow release of active metabolites from a drug-macromolecule complex. The survival of cells in pulmonary and sc tumors after cyclophosphamide treatment has been studied in situ using a biochemical assay, and the influence of environmental factors at the tumor site has been assessed. The pattern of drug distribution has been studied in large sc tumors, and in one tumor line the drug concentration achieved has been related to estimated cell survival.

[Cancer Treat Rep 60:459-464, 1976]

Several reports have suggested that tumors growing at different sites in a host animal may show a differential sensitivity towards cyclophosphamide (CP) (1,2). However, it is not yet clear whether this differential sensitivity results from the attainment of different effective drug concentrations in the tumor cells or from variations in biochemical or physiologic factors between tumors growing at different sites.

In the work reported here the distribution and biochemical effects of CP have been studied in tumors and normal tissues of rats and mice using ^{32}P-labeled CP. A biochemical method for the assessment of tumor cell survival in vivo has been developed and was used to study the relationship between tumor drug concentration and cell survival in mice bearing sc and pulmonary Lewis lung carcinomas.

EXPERIMENTAL METHODS

The animals used in these studies were rats of the highly inbred August strain bearing the BICR/A15 carcinoma and C57BL mice bearing the Lewis lung carcinoma. Single-cell suspensions of Lewis lung carcinoma cells were prepared from pulmonary metastases and inoculated into each recipient mouse by iv and sc injection in order to produce both pulmonary and sc tumors derived from a single cell line in the same host. The subsequent studies were started on the 14th day after inoculation. ^{32}P-CP (Radiochemical Centre, Amersham, England; initial specific activity, 5 millicuries/mmol) was administered by ip injection at dose levels ranging from 100 to 300 mg/kg of body weight. 6-^{3}H-thymidine (Radiochemical Centre, Amersham, England; specific activity, 30 curies/mmol) was injected ip at a dose of 250 microcuries/kg 1 hour prior to the death of the animal. Nucleic acids were extracted by a modified Schmidt-Thannhauser method (3) and assayed by colorimetric (4) or ultraviolet spectrophotometric methods using an appropriate correction for protein contamination (3).

Radioactivity was measured in a liquid scintillation spectrometer, using a scintillant mixture of butyl-PBD (0.6% wt/vol) in toluene-Tergitol TP9 (7:3). For the dual isotope studies, appropriate corrections were made for decay, quenching, and channel crossover.

RESULTS AND DISCUSSION

The gross uptake and the intracellular distribution of ^{32}P-CP have been studied in tumors and normal tissues of both rats and mice. The gross uptake of ^{32}P in the tissues of mice bearing both

[1]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[2]Radiopharmacology Department, Institute of Cancer Research, Sutton, Surrey, England.

*Reprint requests to: Kenneth D. Tew, Division of Biophysics, Radiopharmacology Department, Institute of Cancer Research, The Royal Marsden Hospital, Downs Rd, Sutton, Surrey SM2 5PT, England.

sc and pulmonary Lewis lung carcinomas over a period of 96 hours is summarized in table 1. The data show that, with the exception of the spleen at 12 hours, the level of drug uptake is broadly similar in all the tissues studied. However, the uptake in the pulmonary tumors is significantly greater ($P < 0.001$) than in the sc tumors. The gross uptake of ^{32}P in tissues indicates the total amounts of CP and its metabolites, but gives no indication of the extent to which the drug has interacted with the cellular RNA and DNA. The relative distribution of the ^{32}P-CP among the acid-soluble, RNA, and DNA fractions of the tissues has been studied, and the results obtained with the pulmonary and sc Lewis lung tumors are shown in table 2. The data in this table show that there is a steady and fairly rapid fall in the proportion of acid-soluble ^{32}P which is accompanied by increased association of ^{32}P with RNA and DNA, indicating a progressive increase in nucleic acid alkylation over the period studied.

Since the cytotoxic effects of CP are considered to result from alkylation of DNA, particular attention has been paid to the time course of nucleic acid alkylation in both rat and mouse tissues. The changes in DNA alkylation with time after CP administration in the BICR/A15 rat tumor and in rat liver, spleen, and kidney are illustrated in figure 1; figure 1 also shows the pattern of RNA alkylation in the rat tumor. The data in figure 1 and

TABLE 1.—Gross uptake of ^{32}P-CP in mice bearing pulmonary and sc Lewis lung carcinoma

Tissue	Total ^{32}P-CP (cpm/mg) at various times (hrs) after ^{32}P-CP treatment			
	12	24	48	96
Kidney	1074 ±102	1313 ±50	1099 ±57	868 ±50
Spleen	2638 ±122	947 ±28	894 ±38	955 ±86
Gastrointestinal epithelium	919 ±53	1015 ±32	785 ±66	850 ±70
Liver	1390 ±63	1655 ±50	1273 ±87	1014 ±101
Lewis lung tumor, sc	863 ±41	1199 ±62	1202 ±31	893 ±56
Lewis lung tumor, pulmonary	1020 ±30	1350 ±51	954 ±50	696 ±52

TABLE 2.—Relative distribution of ^{32}P-CP in pulmonary and sc Lewis lung carcinoma in C57BL mice

Fraction	Relative intracellular ^{32}P-CP association (%) at various times (hrs) after drug treatment								
	0.25	0.5	1	2	4	12	24	48	96
Pulmonary tumor									
Acid-soluble	98	—	—	93	—	48	46	34	36
RNA	2.2 ±0.1	—	—	5.5 ±0.2	—	46.3 ±0.9	45.8 ±3	59.0 ±0.6	57.2 ±1
DNA	0.56 ±0.01	—	—	0.83 ±0.06	—	5.4 ±0.2	7.6 ±0.2	6.6 ±0.2	7.3 ±0.6
Sc tumor									
Acid-soluble	—	97.3	96	95.8	90	64	63	50	33
RNA	—	2.6 ±0.15	3.6 ±0.08	4.2 ±0.17	8.9 ±0.5	31.4 ±1.1	32.2 ±3.3	43.6 ±0.6	60.2 ±1.3
DNA	—	0.17 ±0.01	0.43 ±0.02	0.63 ±0.03	1.92 ±0.2	4.02 ±0-2	4.5 ±0.4	6.14 ±0.3	7.04 ±1

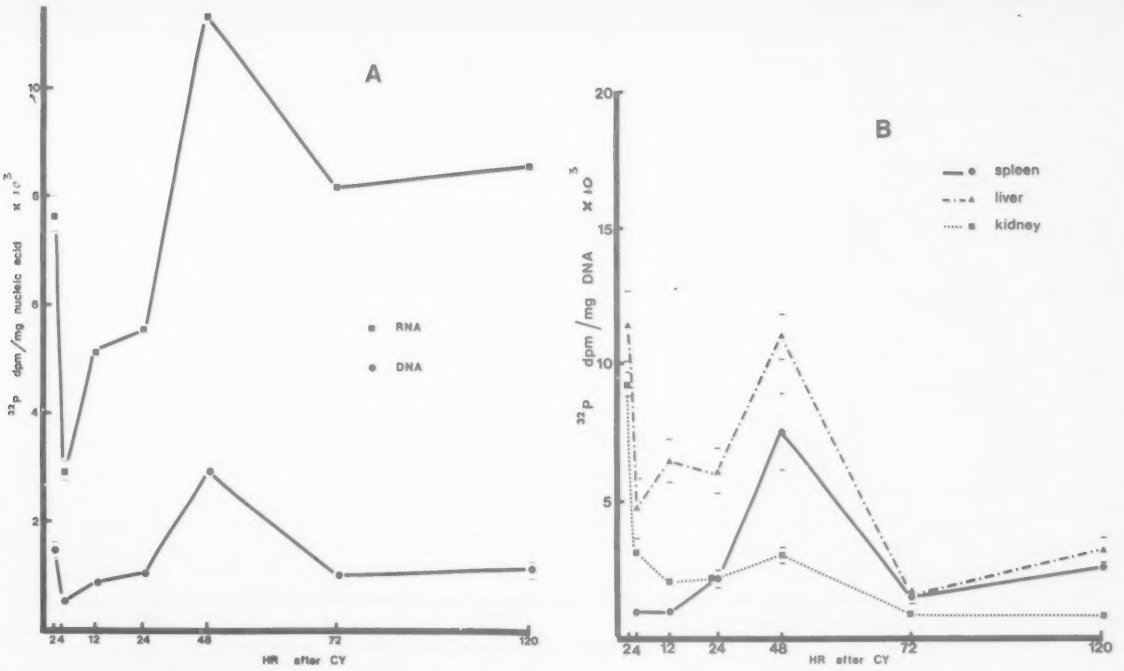


FIGURE 1.—Association of ^{32}P-CP with DNA and RNA in BICR/A15 tumor (A) and with DNA in the liver, spleen, and kidneys of the same rats (B). Each symbol represents the mean ± 1 SEM for 8 animals.

table 2 show that RNA alkylation is more extensive than DNA alkylation. In the rat tumor and liver there is a high initial level of association of ^{32}P with DNA which decreases to a minimum at 4 hours and thereafter increases to reach a maximum at 48 hours. The transient early association of ^{32}P with DNA and RNA may represent ionic bonding of the drug to the nucleic acid rather than true alkylation, and this possibility is being investigated further.

The initial high and transient association of ^{32}P with nucleic acid was not seen in mouse tissues (fig 2) which showed only a steady rise in DNA and RNA alkylation over the first 24 hours after CP administration.

The proportions of the total tissue ^{32}P-CP associated with DNA 12 hours after drug administration in various rat and mouse tissues are compared in table 3. These data show that in both species the tissues most susceptible to damage by CP (tumor, intestinal mucosa, spleen, and bone marrow) show the highest proportions of alkylated DNA, although as is shown by the results in table 1 these tissues do not show higher total uptake of the drug.

The mouse spleen shows an anomalous pattern in relation to both total drug uptake and degree of DNA alkylation (tables 1 and 3). The implantation of Lewis lung carcinoma cells into the C57BL

TABLE 3.—Percentage of total tissue ^{32}P associated with DNA in rat and mouse tissues 12 hrs after ^{32}P-CP treatment

	% of total tissue ^{32}P associated with DNA in—*	
Tissue	Mouse	Rat
Liver	1.8	2.3
Spleen	29.0	3.4
Kidney	1.8	2.6
Gastrointestinal mucosa	2.8	3.2
Bone marrow	—	13.8
BICR/A15 tumor	—	3.4
Lewis lung tumor, sc	4.0	—
Lewis lung tumor, pulmonary	5.4	—

* Values represent means of at least 8 determinations.

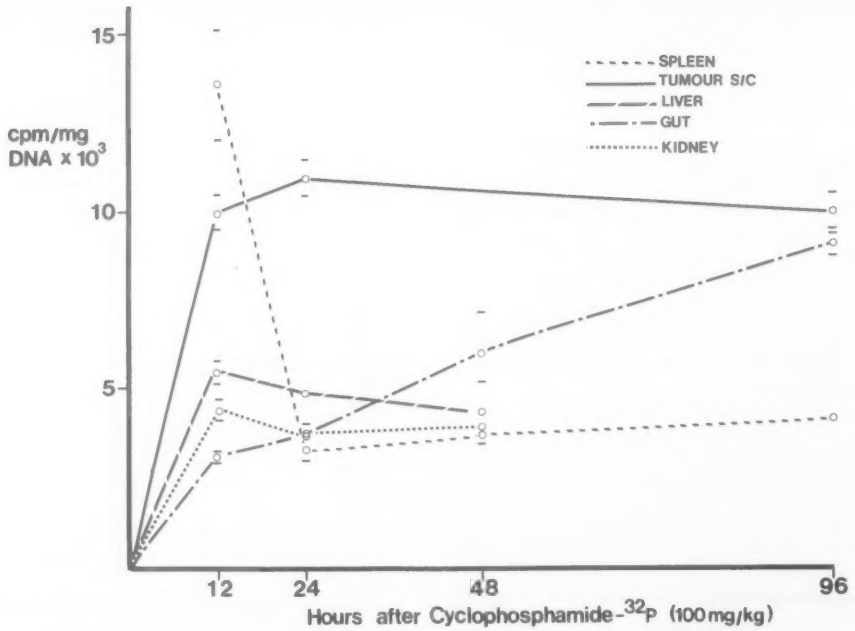


FIGURE 2.—Association of ^{32}P-CP with DNA in sc Lewis lung tumors, spleen, liver, gastrointestinal mucosa, and kidney of C57BL mice. Each point represents the mean of at least 10 determinations ± 1 SEM.

mouse produces a fivefold increase in spleen weight, presumably in response to a tumor antigen, and the enlarged spleen regresses very rapidly after treatment with CP.

These results suggest that alkylation of tumor cell DNA may continue for 24 hours after CP administration in the mouse and for 48 hours in the rat. In vitro assays of clonogenic cells have shown that the survival of B16 melanoma cells removed from the host 2 or 20 hours after CP treatment is similar (Steel GG, personal communication). Since continued DNA alkylation in situ would be expected to lead to greater cell kill after 20 hours, CP must either induce maximum lethal damage to the cell within 2 hours of administration or DNA alkylation must continue in the cells removed at 2 hours for the clonogenic assay. If, as seems likely, the latter does occur, the drug producing the continued alkylation must be present in the cell at the time of its removal from the host. This would suggest that either the active metabolite of CP is only slowly inactivated within the cell, or that there is some intracellular mechanism, possibly involving a drug-macromolecule complex, which causes a slow release of active metabolite over a period of 24-48 hours.

Houghton and Taylor (5) have shown that pulmonary metastases of the Lewis lung tumor have a complex growth pattern, and it was therefore of interest to examine in more detail the influence of the site of tumor growth on the response to CP.

In order to assess the survival of tumor cells after treatment with CP in small tumors, a biochemical method has been developed (Houghton PJ, PhD thesis, University of London, 1975). Changes in the proportion of DNA-synthesizing cells in the tumors are observed by measuring the fractional incorporation (P) of ^{3}H-thymidine into DNA at varying times after drug treatment. P is given by the expression

$$P = \frac{\text{cpm in DNA fraction}}{\text{cpm in total tissue sample}}$$

and is independent of the size of the sample examined or the overall level of ^{3}H-thymidine uptake by the tissue. From serial measurements of P, the time (t) required for P to regain its pretreatment value is obtained and a measure of cell survival, called the equivalent repopulating fraction (ERF), is calculated from the expression

$$\text{ERF} = \tfrac{1}{2}t/c$$

where "c" is the cell cycle time of the tumor.

Using this technique the cell survival has been compared in pulmonary and sc tumors following treatment with CP at doses ranging from 50 to 300 mg/kg. The results are listed in table 4, which also includes comparative data for the sc tumors obtained using an in vitro assay technique (Steel GG, personal communication). These results show that there is a slightly greater degree of cell kill in pulmonary than in sc tumors, and this difference has been found to be reproducible in four separate experiments. For the sc tumors there is good agreement between the results of the in vivo measurement of ERF and the in vitro measurement of the clonogenic fraction of cells within the tumor. The increased sensitivity of pulmonary as compared to sc tumors may be due to either selection of certain sublines of cells which grow well in the pulmonary environment or to greater effective drug concentrations attained in lung tumors. The former possibility seems unlikely since in our studies the tumors were all grown from suspensions of pulmonary tumor cells. The data presented in table 1 show that at 12 and 24 hours after injection the tumor uptake of CP is about 10% higher in pulmonary as compared to sc tumors. However, the proportion of the drug associated with DNA was also higher in the pulmonary tumors (table 2); this may reflect differences in the composition of the tumors since pulmonary tumors have been previously shown to have a higher DNA concentration than sc tumors (5).

The pulmonary tumors examined have been small (< 10 mg) and showed little evidence of necrosis. In contrast, the sc tumors examined have been larger (generally 0.5-1 g, but occasionally up to 3 g) and have contained areas of necrosis. Thus, in these latter tumors uptake of drug into nonviable cells may reduce the overall effect of the drug on the tumor. The distribution of ^{32}P-CP in sc tumors has been examined. Tumors were excised, frozen, and sectioned as illustrated in figure 3, and each segment was assayed for ^{32}P activity. The results obtained with the BICR/A15 rat tumor are illustrated in figure 3, and table 5 contains similar data for sc Lewis lung tumors weighing 2-3 g. In both tumors the inner, frequently necrotic, areas contain less CP than the outer regions. In the Lewis lung tumor it was possible to calculate the expected cell survival corresponding to the actual drug uptake in each segment. The data presented in table 4 show that expected cell survivals vary by factors of up to 10^4 between different segments. Although many of the areas exhibiting low uptake of drug are largely necrotic, there is evidence from ^{3}H-thymidine uptake studies in Lewis lung tumors and from transplantation experiments with other cell lines (6) to suggest that a few viable cells may remain in such areas; thus, because of the lower drug uptake in necrotic areas these cells may have a greater chance of surviving after drug treatment than viable cells in other tumor areas.

There was some indication from our studies that large pulmonary tumors (> 10 mg) were less sensitive to CP than very small tumors (< 2 mg); however, this differential was small compared to that reported by DeWys (1) for the sensitivity of pulmonary and Lewis lung tumors.

The results reported in our study suggest that the greater sensitivity of pulmonary as compared to sc Lewis lung tumors is due to the attainment of slightly greater drug concentrations in the lung tumors. It seems more likely that this difference in uptake is due to the small size of the pulmonary as compared to the sc tumors than to environmental differences between the two sites.

It is probable that the difference in cell kill between the primary and metastatic growths reported by DeWys (1) is due to differences in the cells rather than differences in the site of growth. Our data support the view that there is little or no advantage to be gained in the treatment of lung tumors with CP in comparison to sc or im carcino-

TABLE 4.—Estimated cell survival in pulmonary and sc Lewis lung tumors after treatment with CP

Dose (mg/kg)	Method of estimation		
	Incorporated TdR (equivalent repopulating fraction, sc)	Incorporated TdR (equivalent repopulating fraction, pulmonary)	In vitro assay (clonogenic fraction, sc)
50	1.4×10^{-1}		2×10^{-1}
100	1.7×10^{-2}	9.5×10^{-3}	2×10^{-2}
200	3.5×10^{-4}		1×10^{-4}
300	7.6×10^{-6}	4×10^{-6}	1×10^{-6}

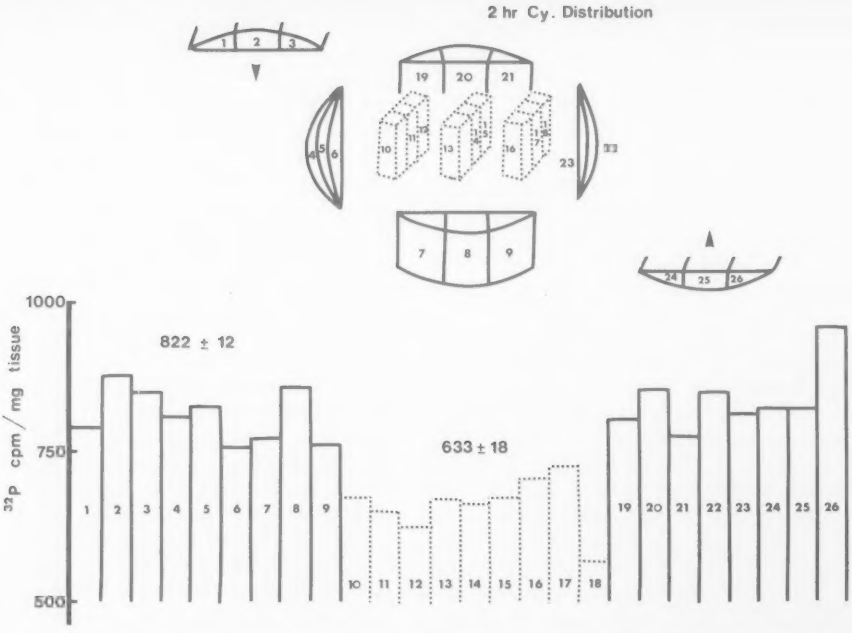


FIGURE 3.—Spatial distribution of ^{32}P-CP in an sc BICR/A15 rat tumor (weight, 10 g). The core of the tumor, represented by the dotted lines (samples 10-18), was almost entirely necrotic. The periphery of the tumor (samples 1-9 and 19-26) contained some areas of necrosis. Figures above the bars represent the mean ^{32}P activity (± 1 SEM) in the periphery and core of the tumor.

TABLE 5.—Variation in ^{32}P-CP concentration and cell survival in a 2-3-g sc Lewis lung tumor from a C57BL mouse

Tumor cross-section		% of pulmonary drug concentration		Corresponding cell survival	
		Outside	Inside	Outside	Inside
End	A	100 ± 5	64 ± 6	4×10^{-5}	2×10^{-4}
↑	B	87 ± 10	33 ± 7	2×10^{-5}	2×10^{-2}
	C	71 ± 5	30 ± 5	5×10^{-5}	3×10^{-2}
	D	54 ± 6	35 ± 5	6×10^{-4}	1×10^{-2}
Center	E	62 ± 5	40 ± 6	2×10^{-4}	5×10^{-3}
	F	49 ± 6	21 ± 4	2×10^{-3}	8×10^{-2}
	G	51 ± 6	23 ± 5	2×10^{-3}	3×10^{-2}
↓	H	— —	— —	—	—
	I	74 ± 5	109 ± 10	5×10^{-5}	2×10^{-6}
End	J	90 ± 5	90 ± 5	6×10^{-6}	1×10^{-5}

mas unless the metastatic pulmonary carcinomas are derived from a hypersensitive cell line.

REFERENCES

1. DEWYS WD. Studies correlating the growth rate of a tumor and its metastases and providing evidence for tumor-related systemic growth-retarding factors. Cancer Res 32:374-379, 1972.
2. SIMPSON-HERREN L, SANFORD AH, and HOLMQUIST JP. Cell population kinetics of transplanted and metastatic Lewis lung carcinoma. Cell Tissue Kinet 7:349-361, 1974.
3. MUNRO HN, and FLECK A. The determination of nucleic acids. Meth Biochem Anal 14:113-176, 1968.
4. BURTON K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem J 62:315-323, 1956.
5. HOUGHTON PJ, and TAYLOR DM. Environment and tumor growth. (Abstr) Br J Cancer 31:266, 1975.
6. GRISWOLD DP, JR, SCHABEL FM, JR, WILCOX WS, ET AL. Success and failure in the treatment of solid tumors. 1. Effects of cyclophosphamide (NSC-26271) on primary and metastatic plasmacytoma in the hamster. Cancer Chemother Rep 52:345-387, 1968.

Studies on the Enhancement by Cyclophosphamide (NSC-26271) of Artificial Lung Metastasis After Labeled Cell Inoculation[1]

J. de Ruiter, * T. Smink, and L. M. van Putten[2,3]

SUMMARY

In a mouse model the retention of ^{125}I-5-iodo-2′-deoxyuridine-labeled tumor cells in the lung after iv injection was compared with the formation of tumor colonies in the lung 15 days after injection. The modification in lung retention after treatment with cyclophosphamide (CP), isophosphamide, *Corynebacterium parvum*, and heparin paralleled the changes in lung-colony numbers by these treatments. Since the modifications could be identified as early as 1 hour after iv administration of tumor cells, further evidence was obtained for the conclusion reached earlier that the modification of lung metastases by CP and *Corynebacterium parvum* is not due to immunologic mechanisms. Comparison with the lung retention of heat-killed tumor cells and living embryonic cells showed that CP induced an increased retention of all three cell types. In contrast, *Corynebacterium parvum* decreased the retention of living tumor cells, but failed to modify the retention of dead tumor or living embryonic cells. Heparin decreased the retention of living and dead tumor cells, but did not affect the retention of living embryonic cells. Since no similar cell types were affected by the various treatments it seems likely that different mechanisms are involved. Apparently CP decreases the nonspecific resistance against the lodging and growth of any type of cell in the lung.

[Cancer Treat Rep 60:465-470, 1976]

In a mouse model, the pretreatment of animals with cytostatic drugs enhanced the formation of metastatic lung nodules after iv injection of osteosarcoma cells (1). This effect could not be attributed to immunologic mechanisms, since neither immunosuppression nor immunization changed the number of lung nodules significantly.

Cyclophosphamide (CP) enhanced the formation of metastatic lung nodules by a factor of 30->1000, whereas other cytostatic drugs, including the CP congeners isophosphamide (IP) and trophosphamide,[4] were active only by a factor of between two and 12. *Corynebacterium parvum*,[5] a macrophage stimulant (2-4), is highly effective in suppressing lung nodule formation (1,5,6). Anticoagulant treatment of mice at the time of tumor inoculation also diminished the formation of lung nodules (table 1). The interference of coagulation mechanisms with the development of blood-borne metastases has been described for many tumors (7-12).

In order to provide more information on the mechanisms involved in the enhancement of lung metastases by CP, this paper presents an analysis of the differences in the retention of osteosarcoma cells labeled with ^{125}I-5-iodo-2′-deoxyuridine (^{125}IUDR)[6] in the lungs of mice pretreated with CP according to a method described by Brown (12). He observed that increased formation of lung metastases after local lung irradiation was reflected by an increase in the retention of ^{125}IUDR-labeled tumor cells in the lung (13). Comparable results were obtained after CP treatment in our study. In order to investigate whether the early increase in retention of osteosarcoma cells was associated

[1]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate. Supplied by Asta-AG, Brackwede, Germany.

[2]Radiobiological Institute TNO, Rijswijk, The Netherlands.

[3]The authors thank C. Zurcher for helpful discussions and H. M. Jense, M. van der Sman, and E. J. van der Reijden for the preparation of this manuscript.

[4]Supplied by Asta-AG, Brackwede, Germany.

[5]Supplied by Wellcome Foundation, Beckenham, Kent, England.

*Reprint requests to: J. de Ruiter, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk, The Netherlands.

[6]Supplied by Amersham, Buckinghamshire, England.

TABLE 1.—Effect of anticoagulant administration to recipients on the frequencies of lung nodule formation after iv injection of osteosarcoma cells

Anticoagulant	Dose	Lung nodule formation efficiency*
Warfarin (4 days before tumor injection)	†	0.12-0.23
Heparin (1 hr before tumor injection)	150 IU	0.02
Antiplatelet globulin (24 hrs before tumor injection)	2 mg	0.02-0.02

*Each figure represents the results obtained by comparing the number of nodules in 5 treated mice with the number in 5 or more control mice that had received samples of the same tumor-cell suspension.

†7.5 μg/ml given in drinking water for 4 days, followed by 5 μg/ml given for 6 days.

with the viability of the tumor cells or with some specific characteristics of tumor cells, the retention of heat-killed osteosarcoma cells and living isogenic embryonic cells in the lung was followed. The modification in the retention of these cells by CP was compared with the modification after IP, heparin,[7] and *Corynebacterium parvum* treatment to determine whether a similar mechanism was involved.

MATERIALS AND METHODS

Mice.—Studies with tumor cells were performed with the C22LR mouse osteosarcoma. This tumor originated in a (C57BL/Rij × CBA/Rij)F_1 hybrid mouse and was serially transplanted in similar mice or stored in liquid nitrogen. The tumor is nonantigenic in this host and only passages 76-83 were used for experiments. Embryonic cells were obtained from all tissues of 15-17-day-old embryos of C57BL/KA mice and injected into isogenic mice. Male mice 10-14 weeks of age were used for all studies.

Cell labeling and injection.—The clearance of labeled cells from the lung was investigated according to Brown (12). Donor mice bearing osteosarcoma tumors or embryos were given injections (four ip or four sc) of 0.5 microcurie/g body weight of ^{125}IUDR (1-6 millicuries/mg and, in later experiments, 80-120 millicuries/mg). The tracer was injected at 8- or 16-hour intervals. One hour after the last injection, the mice were killed and a cell suspension was prepared according to the method of Reinhold (14). However, two additional washings were performed to remove any nonbound radioactivity. After this, 2.5-5 million (eosin-excluding) labeled cells in 0.5 ml Hanks' balanced salt solution were injected iv into recipient mice that had received 0.1% potassium iodide in their drinking water for at least 1 day before injection. If heat-killed cells were injected, the labeled cells were heated for 30 minutes at 56°C and washed once afterwards. In one experiment, the osteosarcoma cells were labeled with ^{51}Cr according to a method described by Fisher and Fisher (15). Briefly, the osteosarcoma cells were incubated with $Na_2{}^{51}CrO_4$ for 1 hour at 37°C; the free chromium was reduced with 100 mg of ascorbic acid and the cells were washed four times afterwards.

Pretreatment of mice with various agents.—The agents (except heparin) were given 2 days before the cell injections unless stated otherwise. Heparin was injected 15 minutes before the cell injections. The doses given were: (*a*) 250 mg/kg CP sc, (*b*) 200 mg/kg IP sc, (*c*) 0.35 mg/mouse *Corynebacterium parvum* iv, and (*d*) 150 international units heparin ip. Rabbit antimouse platelet globulin[8] was given in a dose that caused thrombocytopenia.

Measurement of lung clearance of labeled cells.—At several intervals after injection of labeled cells, groups of two to five mice were killed, their lungs were removed, and the radioactivity was assayed in vitro in a well-type gamma-counter.

RESULTS

The effect of pretreatment of mice with CP, IP, heparin, and *Corynebacterium parvum* on the lung clearance of iv injected labeled osteosarcoma cells is shown in figure 1. With the exception of the mice treated with heparin, essentially all osteosarcoma cells were arrested initially in the lungs. Both the initial rapid loss of a large proportion of cells during the first 24 hours after injection and the slow exponential loss of the rest of the cells from then on could be modified by the different types of treatments.

[7]Thromboliquine. Supplied by Organon Oss, The Netherlands.

[8]Supplied by A. Nakeff, Division of Cancer Biology, The Mallinckrodt Institute, St. Louis, Mo.

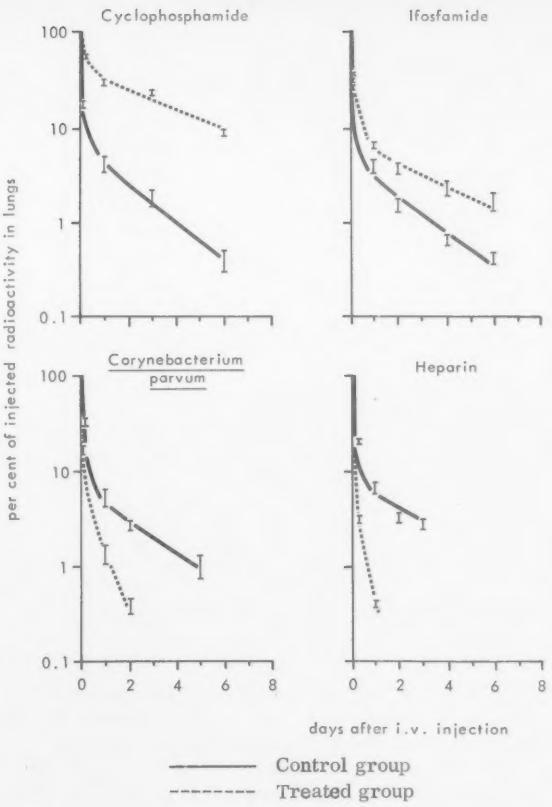


FIGURE 1.—Effect of pretreatment of mice with various agents on the retention of iv injected ^{125}IUDR-labeled osteosarcoma cells in the lungs. Bars indicate mean ± SE; straight lines were calculated by the least squares method fitting the experimental points.

CP treatment markedly increased the retention of tumor cells in the lungs. The results of a typical experiment are presented in table 2. In addition, the exponential loss of cells from about 1 day after injection took place at a slower rate than in the control mice. IP treatment influenced the cell loss in the same way, but to a much lesser extent. On the other hand, treatment with *Corynebacterium parvum* or heparin decreased the retention of tumor cells, which was already significantly different from the controls at 1 hour after injection; at 3 days after injection, radioactivity could no longer be measured (< 0.1%). These results show that the increase in tumor cell retention in the lung after CP and IP treatment correlates with the reported increase in the formation of lung metastases. The decrease after heparin (table 1) and *Corynebacterium parvum* reflects the decreased formation of lung metastases (1).

TABLE 2.—Effect of CP on the lung retention of ^{125}IUDR-labeled living osteosarcoma cells

Time after injection	% of injected radioactivity* Control	CP† (250 mg/kg)
5 mins	94.6 ± 2.5	97.4 ± 2.2
1 hr	74.4 ± 1.3	87.3 ± 1.1‡
3 hrs	32.7 ± 1.8	67.4 ± 2.4‡
6 hrs	16.8 ± 1.4	60.8 ± 1.4‡
24 hrs	5.0 ± 0.4	48.2 ± 2.1‡
2 days	2.7 ± 0.1	41.1 ± 2.4‡
5 days	1.1 ± 0.2	23.9 ± 1.5‡

*Mean ± SE for 3-5 mice.
†Administered 48-52 hrs before iv inoculation of osteosarcoma cells.
‡$P < 0.05$ (student's t test).

Lung Retention of Labeled Heat-Killed Osteosarcoma and Living Embryonic Cells

Since the mechanisms involved in enhancement of lung metastases may be associated with some properties of the osteosarcoma cells, the lung retention of heat-killed osteosarcoma cells and of isogenic embryonic cells was also followed. The results in control mice are presented in figure 2 and it is evident that the retention of the other cell types is decreased when compared to the retention of living osteosarcoma cells. Furthermore, killing the cells by heat before injection decreased the retention of both osteosarcoma and embryonic cells. The modifications in lung retention of these cell types after treatment with CP, IP, *Corynebacterium parvum*, and heparin are presented in table 3. The results are expressed as the ratio of the radioactivity in the lungs of treated mice over control mice. CP increased the retention of all cell types. Heparin decreased the retention of living and dead osteosarcoma cells, whereas IP and *Corynebacterium parvum* systematically affected only the retention of living osteosarcoma cells.

Lung Retention of ^{51}Cr-Labeled Heat-Killed Osteosarcoma Cells

In a number of studies, ^{51}Cr-labeled tumor cells were used to study the cell retention in various organs (5,15) or cell death (16).

In our study, the retention of radioactivity in the lungs was compared after injection of heat-killed ^{51}Cr- and ^{125}IUDR-labeled osteosarcoma cells in control mice. The results are presented in table 4. Initially, only half of the radioactivity of the

TABLE 3.—Effect of various agents on the lung retention of various ^{125}IUDR-labeled cells

	Ratio of lung retention (treated mice/control mice) at various times after injection				
	5 mins	1 hr	3 hrs	6 hrs	24 hrs
CP					
Living osteosarcoma cells	1.0	1.2*	2.1*	3.3*	9.9*
Dead osteosarcoma cells	1.1*	1.6*	1.8*	2.8*	2.7*
Living embryonic cells	1.2	1.8*	2.5*	3.3*	4.1*
IP					
Living osteosarcoma cells	1.0	1.0	1.3*	1.4*	1.7*
Dead osteosarcoma cells	1.0	0.9	1.3*	0.8*	1.0
Living embryonic cells	1.0	0.9	1.3	1.0	1.3
Corynebacterium parvum					
Living osteosarcoma cells	0.9	0.8*	0.5*	0.6*	0.2*
Dead osteosarcoma cells	1.0	1.1	1.6	1.0	1.2
Living embryonic cells	1.0	0.9	0.9	0.7	1.1
Heparin					
Living osteosarcoma cells	0.8*	0.8*	0.8*	0.5*	0.2*
Dead osteosarcoma cells	0.7*	0.6*	0.2*	0.3*	
Living embryonic cells	0.7*	1.1	1.0	0.7	1.0

*$P < 0.05$ (student's t test).

^{51}Cr-labeled cells was retained in the lung in contrast to 100% retention of ^{125}IUDR-labeled cells. This discrepancy may be due to a high concentration of nonincorporated ^{51}Cr in the injection suspension, which will not be retained in the lung (16). The results at later intervals after injection show that the retention of radioactivity was much higher for ^{51}Cr-labeled cells than for ^{125}IUDR-labeled cells.

DISCUSSION

The results of studies with labeled cells seem to parallel the results of lung-colony counts (table 5). The differences in retention of osteosarcoma cells in the lungs of treated mice are shown as early as 1 hour after the first contact with these cells, confirming an earlier conclusion (1) that modification of lung metastases by CP and *Corynebacterium parvum* is not due to immunologic mechanisms.

In the lung-colony assay (1), pretreatment with CP enhanced the formation of lung nodules by a factor of 30->1000. Since the lung nodules are counted 15 days after injection, the difference in retained radioactivity must be compared at this time.

In the lung retention curves of living osteosarcoma cells, the rapid fall in the lung radioactivity is followed by a slow exponential loss of radioactivity. Since the slope of the exponential loss after CP treatment is different from the control group, comparison of retained radioactivity must be performed by extrapolation to Day 15. This would

TABLE 4.—Difference in retained radioactivity in the lung after injection with ^{51}Cr- and ^{125}IUDR-labeled dead osteosarcoma cells

	% of injected radioactivity	
Time after injection	^{51}Cr-labeled cells (mean ± SE)	^{125}IUDR-labeled cells (mean ± SE)
5 mins	56.0 ± 0.5	100.2 ± 0.9
24 hrs	10.3 ± 0.2	0.3 ± 0.1
2 days	5.3 ± 0.2	
5 days	2.9 ± 0.2	

TABLE 5.—Modification of labeled cell retention in the lung and lung nodule formation after iv injection of osteosarcoma cells

	Ratio (treated mice/control mice) of—	
Pretreatment	Lung radioactivity (at 3 days)	Lung nodules (at 15 days)
CP	11	158->1000
IP	3	3-9
Heparin	<0.03	0.02
Corynebacterium parvum	<0.02	<0.01

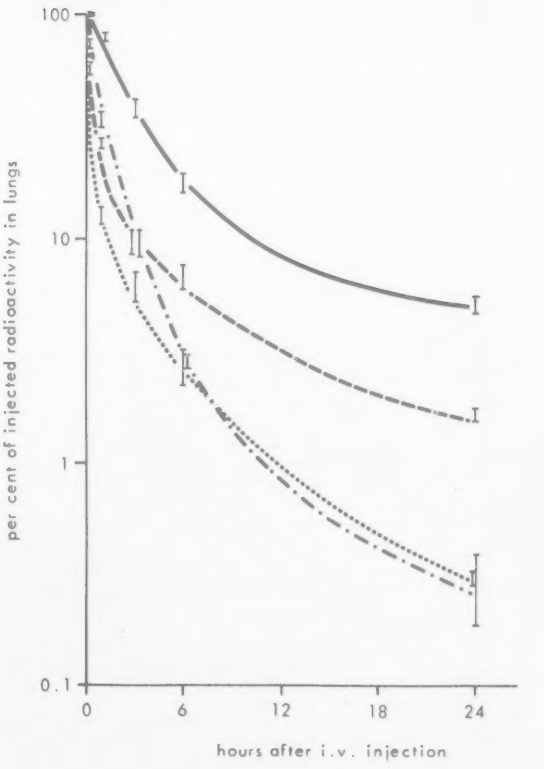


FIGURE 2.—Retention of various types of ^{125}IUDR-labeled cells in the lungs of control mice after iv injection. Bars indicate mean ± SE; lines are drawn by eye.

result in about 180 times more radioactivity in the CP group than in the control group, which correlates rather well with the enhancement factor of 30->1000 in lung nodule formation (1). Furthermore, the smaller differences in retention of radioactivity after IP correlate well with the lower enhancement factor.

However, in the interpretation of the labeling results, it must be considered that the decrease in retention of radioactivity might express the loss of cells which are not destined to initiate metastases, since the cloning efficiency of the osteosarcoma cells is very low (about 0.1%). For that reason, differences in the exponential slope are rather difficult to interpret.

After heparin and *Corynebacterium parvum* treatment, no radioactivity could be detected a few days after injection of tumor cells. This is in agreement with the decreased formation of lung metastases. Since the lower limit of detection of radioactivity is at about 0.1% of the injected radioactivity (5×10^3 cells), the few cells necessary to develop metastases were no longer detected.

The modifications in lung retention of living osteosarcoma cells after various treatments were compared with the modifications in lung retention of dead osteosarcoma and living embryonic cells. CP increased the retention of all three cell types, although the extent of the increase 24 hours after injection was smaller for dead osteosarcoma and living embryonic cells than for living osteosarcoma cells. The effects of IP on living osteosarcoma cells were smaller than for CP, as was also shown for the effect on lung nodule numbers. Therefore, it is not surprising that no effects on the other cell types could be recognized.

Since anticoagulation with warfarin, heparin, and antiplatelet globulin decreased the formation of lung metastases by factors between ten and 50, it is possible that the enhanced formation of lung metastases after CP treatment is due to increased coagulation. However, if coagulation mechanisms were involved, it would be expected that heparin treatment would have modified the lung retention of similar cell types as did CP, although in the opposite direction. This was not observed; heparin treatment decreased the lung retention of living and dead osteosarcoma cells only. No difference was observed with embryonic cells, although such a difference could have been demonstrated as shown for killed cells (fig 2). This finding might indicate that the effect of CP is not mediated by coagulation mechanisms. *Corynebacterium parvum* decreased the retention of living osteosarcoma cells but failed to modify the retention of dead osteosarcoma and living embryonic cells. Since this decrease was rather marked 24 hours after injection of living osteosarcoma cells, it seems unlikely that a similar decrease with dead osteosarcoma and living embryonic cells might have remained unnoticed. For that reason, these results indicate that the effect of *Corynebacterium parvum* is specifically associated with the viability of the cell and with specific characteristics of tumor cells. In contrast to this, the effect of CP does not differentiate among the various cell types. In conclusion, there is no evidence that a similar mechanism(s) is involved in the modification of lung metastases in our mouse model by treatment with CP, *Corynebacterium parvum*, or heparin.

Furthermore, the preferential use of the ^{125}IUDR label over the ^{51}Cr label has been shown. ^{125}IUDR is poorly reutilized after cell lysis of labeled cells (17-19), so the decrease in radioactivity in the lung provides an estimate of the loss or death of ^{125}IUDR-labeled cells from the lung. This is confirmed in our study by the differences in retention of radioactivity after the injection of dead ^{51}Cr-labeled cells. This difference is probably due to reincorporation of ^{51}Cr label in normal cells. These findings confirm that the retention of ^{51}Cr does not reflect the real retention of cells (12,19).

REFERENCES

1. VAN PUTTEN LM, KRAM LKJ, VAN DIERENDONCK HHC, ET AL. Enhancement by drugs of metastatic lung nodule formation after intravenous tumour cell injection. Int J Cancer 15:588-595, 1975.
2. CASTRO JE. The effect of *Corynebacterium parvum* on the structure and function of the lymphoid system in mice. Eur J Cancer 10:115-120, 1974.
3. WOLMARK N, and FISHER B. The effect of a single and repeated administration of *Corynebacterium parvum* on bone marrow macrophage colony production in syngeneic tumor-bearing mice. Cancer Res 34:2869-2872, 1974.
4. MCBRIDE WH, JONES JT, and WEIR DM. Increased phagocytic cell activity and anaemia in *Corynebacterium parvum* treated mice. Br J Exp Pathol 55:38-46, 1974.
5. BOMFORD R, and OLIVOTTO M. The mechanisms of inhibition by *Corynebacterium parvum* of the growth of lung nodules from intravenously injected tumour cells. Int J Cancer 14:226-235, 1974.
6. MILAS L, HUNTER N, and WITHERS HR. *Corynebacterium granulosum*-induced protection against artificial pulmonary metastases of a syngeneic fibrosarcoma in mice. Cancer Res 34:613-620, 1974.
7. WOOD S, JR, YARDLEY JH, and HOLYOKE ED. The relationship between intravascular coagulation and the formation of pulmonary metastases in mice injected intravenously with tumor suspension. Proc Am Assoc Cancer Res 2:260, 1957.
8. GASIC GJ, GASIC TB, and STEWART CC. Antimetastatic effects associated with platelet reduction. Proc Natl Acad Sci USA 61:46-52, 1968.
9. AGOSTINO D, and CLIFFTON EE. Trauma as a cause of localization of blood-borne metastases: preventive effect of heparin and fibrinolysin. Ann Surg 161:97-102, 1965.
10. HILGARD P. Der einfluss von Heparin, Plasmin, Trypsin, Protamin und epsilon-Amino-Kapronsäure auf die Klebrigkeit und Aggregation von Aszitерzellen in vitro. Arch Geschwulstforsch 38:27-33, 1971.
11. THORNES RD, EDLOW DW, and WOOD S, JR. Inhibition of locomotion of cancer cells in vivo by anticoagulant therapy. Johns Hopkins Med J 123:305-316, 1968.
12. BROWN JM. A study of the mechanisms by which anticoagulation with warfarin inhibits blood-borne metastases. Cancer Res 33:1217-1224, 1973.
13. BROWN JM. The effect of lung irradiation on the incidence of pulmonary metastases in mice. Br J Radiol 46:613-618, 1973.
14. REINHOLD HS. A cell dispersion technique for use in quantitative transplantation studies with solid tumours. Eur J Cancer 1:67-71, 1965.
15. FISHER B, and FISHER ER. The organ distribution of disseminated ^{51}Cr-labeled tumor cells. Cancer Res 27:412-420, 1967.
16. DE BRABANDER M, AERTS F, and BORGERS M. The influence of a glucocorticoid on the lodgement and development in the lungs of intravenously injected tumour cells. Eur J Cancer 10:751-755, 1974.
17. HEINIGER HJ, FEINENDEGEN LE, FRIEDRICH G, ET AL. Regional reutilization of thymidine in the normal mouse. Kernforschungsanlage Jülich, No. 679, 1970.
18. DETHLEFSEN LA. An evaluation of radioiodine-labeled 5-iodo-2′-deoxyuridine as a tracer for measuring cell loss from solid tumors. Cell Tissue Kinet 4:123-138, 1971.
19. FIDLER IJ. Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with ^{125}I-5-iodo-2′-deoxyuridine. J Natl Cancer Inst 45:773-782, 1970.

Distinctive Effects of Mechlorethamine (NSC-762), Cyclophosphamide (NSC-26271), and BCNU (NSC-409962) on Transcription in Ehrlich Cells[1,2]

R. J. Rutman* and N. G. Avadhani[3]

SUMMARY

Comparisons have been made between mechlorethamine, cyclophosphamide (CP), and BCNU as to the effects of chemotherapeutic drug levels on the transcription of RNA in vivo in Ehrlich cells. Mechlorethamine causes a dose-dependent depression in the synthesis of large Hn RNA transcripts in the nucleus and in the amount of large polysomal mRNA in the cytoplasm without apparent disturbance of polyadenylation and transport. Because of compensating increases in smaller Hn RNA and mRNA chains there is no inhibition of total RNA synthesis. CP seems to accelerate total Hn RNA and mRNA formation but inhibits polyadenylation. CP also produces an excess of short chains with only a minor depression of the synthesis of longer transcripts and messages. BCNU inhibits both RNA synthesis and polyadenylation but without disturbance of the size distribution of the polynucleotide chains. The results with mechlorethamine, in particular, are not easily explained on the basis of premature chain termination at the sites of DNA alkylation.

[Cancer Treat Rep 60:471-476, 1976]

Although the effect on RNA synthesis resulting from damage to DNA has been the object of numerous studies (1), detailed information relating to mechanism remains quite limited (2). This is particularly true of the effects on transcription in eukaryotic cells of drugs binding to DNA. As a result, the possible importance of such effects in the action of alkylating agents cannot be evaluated nor can the nature of the effects be used to compare alkylating agents having different biologic activities.

In this report, we have followed up previously published observations (3,4) on the effects of drugs on transcription in tissue culture cells by extending the work to an in vivo system at chemotherapeutic dose levels of mechlorethamine, cyclophosphamide (CP), and BCNU.

Pronounced differences are noted between BCNU and either mechlorethamine or CP and an overall similarity is observed in the effects of CP and mechlorethamine. While our findings tend to confirm previously reported work (3,4), the differences between nitrogen and phosphoramide mustards may not be insignificant.

MATERIALS AND METHODS

Ehrlich ascites hypotetraploid cells were grown ip in Swiss mice. Cells taken after 6-7 days' growth were used for experimental purposes. Mechlorethamine and CP were given in distilled water and BCNU was given as a diluted ethanol solution. After drug injection the animals were killed. The cells were then removed, diluted threefold with Locke-Ringer medium to about 10^8 cells/ml, and pulsed with ^{3}H-cytidine and ^{3}H-adenosine (2-3 millicuries/ml; ~30 millicuries/mmol). Following incubation, polysomes, polysomal mRNA, and nuclear Hn RNA were prepared.

[1]Supported by Public Health Service grant CA-05295 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Mechlorethamine: CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-nitroso-.

[3]Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

**Reprint requests to:* Dr. R. J. Rutman, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa 19174.

Hn RNA was prepared essentially according to Penman et al (5). Nuclei were prepared and suspended in 0.6 M KCl containing 30 mM tris-HCl (pH 7.5), 5 mM Mg $(CH_3COO)_2$, and 7 mM mercaptoethanol. The suspension was shaken at 37°C with 40 mg/ml DNase (RNase free, Worthington, Freehold, NJ) for 10 minutes, diluted with distilled water (1:1), and made 0.5% with respect to sodium dodecyl sulfate. Two volumes of phenol-chloroform containing 0.1% of 8-hydroxyquinoline were added and the mixture was shaken for 15 minutes at 50°C. The phases were then separated, the phenolic phase was washed, the aqueous phases were combined, and the RNA was precipitated with ethanol-$K(CH_3COO)$. Stored RNA was protected with 0.05% diethylpyrocarbonate.

Cytoplasmic polyribosomes were prepared essentially according to a published method (6). mRNA was released by puromycin and purified by a modified procedure (7,8).

Poly A containing mRNA and Hn RNA was determined by the oligo(dT)-cellulose column procedure (9) as applied by Avadhani et al (8).

Sucrose gradient runs were calibrated with 18S, 28S, and 45S ribosomal RNAs for each condition of centrifugation. Histograms were constructed and used to estimate length distributions using the analysis that was applied to HeLa cells (10). The control patterns obtained for Ehrlich cells were essentially the same as those obtained for HeLa cells (10). In addition, corresponding size distribution patterns are obtained by gel electrophoresis and by sucrose centrifugation.

RESULTS

We have examined the results of treatment of sensitive and resistant Ehrlich ascites tumors with mechlorethamine over a wide dose range. In this report we compare the effect on transcription of chemotherapeutic doses of mechlorethamine with those of CP and BCNU. From table 1, it can be seen that at the chemotherapeutic level of 1.0 mg/kg, mechlorethamine does not inhibit Hn RNA synthesis, nor are the usual quantitative parameters of polyadenylation disturbed. In contrast, a toxic dose of mechlorethamine (6.0 mg/kg) shows both significant overall inhibition as well as alterations in the extent of polyadenylation. A low, chemotherapeutic dose of CP appears to stimulate incorporation of radioactivity into Hn RNA but this is accompanied by disturbances in the pattern of polyadenylation. There is a decrease in the fraction of poly A(+) Hn RNA. Since the poly A content of the oligo(dT)-bound fraction appears normal, the data imply that fewer transcripts have been polyadenylated. In the case of BCNU, the effects on polyadenylation are similar, but are accompanied by an inhibition of Hn RNA synthesis. In table 2, the effects of these changes on polysomal mRNA can be examined. As in the case of Hn RNA synthesis, there is an inhibition of mRNA synthesis by BCNU, a stimulation by CP, and a normal rate is observed in the presence of a low dose of mechlorethamine. The polyadenylation picture for the mRNA is the same as that of the Hn RNA. In the presence of both CP and BCNU, relatively less of the mRNA is bound to oligo(dT), and there is less RNase-resistant material in the mRNA fraction. The apparently normal percentage of poly A in the oligo(dT)-bound fraction is to be expected in view of the constancy of this feature in the Hn RNA. These data imply that the transport of a greater quantity of poly A(-) material to the cytoplasm has occurred after exposure to CP and BCNU.

Figures 1-3 present the sucrose gradient length distribution profiles of the Hn RNA and mRNA from control and treated cells. The striking feature in figures 1 and 2 for low doses of mechlorethamine (1.0 mg/kg or less) is the significant reduction in the percentage of large transcripts and mRNAs. Whereas 40% of the control Hn RNAs are in the size class $> 7.5 \times 10^3$ ribonucleotides, only half as much material is present in the mechlorethamine-treated preparations. As a result of these changes in nucleoplasmic transcription, the polysomes show an almost total absence of mRNAs $> 4 \times 10^3$ nucleotides in length, but a compensatory accumulation of chains $< 2 \times 10^3$ nucleotides in length. We have studied these effects in the mechlorethamine dose range of 0.4-20 mg/kg. The same qualitative picture applies from the lowest to the highest dose, except that at doses of 6 mg/kg or greater there is also an increasing inhibition of overall RNA syntheses. The results for CP (fig 3) are interesting; with regard to Hn RNA there appears to be an increase in small transcripts. This is accompanied by a moderate decline in all other fractions rather than in only the longest fraction. These features are reproduced in the mRNA patterns. Thus the action resembles but is not identical with that of mechlorethamine. In contrast, BCNU has no significant effect on the size-distribution patterns of Hn RNA transcripts or mRNAs.

DISCUSSION

A detailed comparison of the effects of the three drugs (mechlorethamine, CP, and BCNU) on transcription must await the completion of dose and

TABLE 1.—Effects of alkylating chemotherapeutic agents on Hn RNA synthesis and polyadenylation in Ehrlich cells*

Drug	Dose (mg/kg)	Relative specific activity of Hn RNA	Fraction bound to oligo(dT)	Poly A in—	
				Total Hn RNA	Bound Hn RNA
Control	—	1.00	17.2%	1.10%	6.5%
			% of control		
Mechlorethamine	1.0	0.95	99	95	97
CP	60	1.12	70	74	98
BCNU	30	0.72	69	75	104

*Ehrlich ascites cells treated in vivo: mechlorethamine (30 mins), CP and BCNU (120 mins). Cells were removed and pulsed with ^{3}H-cytidine and ^{3}H-adenosine for 45 mins and Hn RNA was isolated as described in Materials and Methods.

time studies on CP and BCNU; such data have already been obtained on mechlorethamine. However, there is preliminary evidence of differences (table 3). It is clear that the effects of mechlorethamine and CP are different from those of BCNU, confirming the observations of Abelson et al (3). However, although these authors observed extensive inhibition of the processing and transport of mRNA (as judged by the oligo[dT]-bound cytoplasmic fraction), our own data show that both Hn RNA and mRNA are affected in similar ways making it more difficult to detect effects on processing and transport alone. It appears that Abelson's results were obtained under conditions of extensive inhibition; ours were obtained at barely significant levels of inhibition.

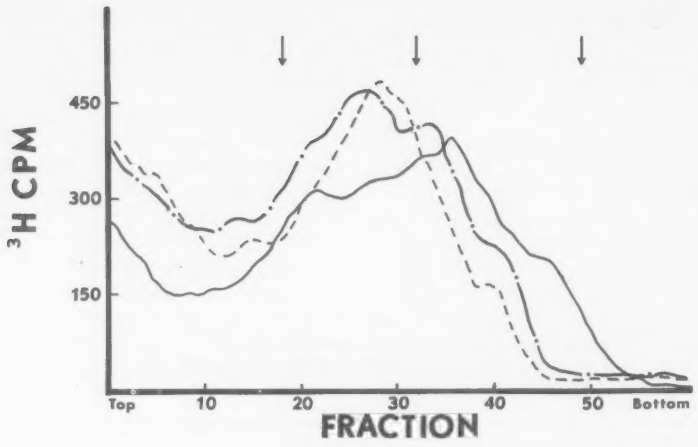
The comparison with CP is of interest because there is a close similarity to the effects of mechlorethamine; if this is true then the quantitative results indicate that 60 mg/kg of CP is approximately equivalent to 0.4 mg/kg of mechlorethamine. There is also the possibility, however, that the increase in small transcripts and mRNAs reflects the increase in total synthesis coupled with a small effect on the size distribution. However, the interference with normal polyadenylation at least indicates that the CP effect is more complicated than that of mechlorethamine. Similarities observed between mechlorethamine and CP are in keeping with their well-known cross resistances (11).

It is of some importance to note that if the effects of mechlorethamine and CP are due to damage to the template function of the DNA this is also unlikely to be a simple effect (eg, premature chain termination at the site of alkylated bases or alkylation of the 3′ end of the nascent transcripts [3]). At doses of mechlorethamine in the range of 0.4-1.0 mg/kg, the frequency of alkylation is 4-10/10^6 deoxynucleotides. About 70% of these are monoalkylations at the N_7-position of guanine (12,13).

TABLE 2.—Effects of alkylating chemotherapeutic agents on polysomal mRNA synthesis and polyadenylation in Ehrlich cells*

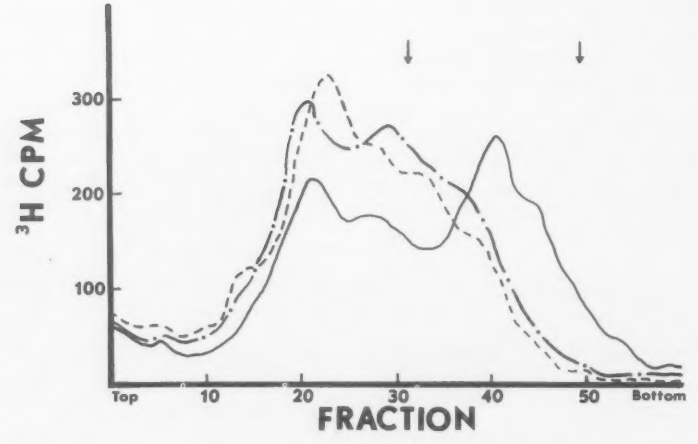
Drug	Dose (mg/kg)	Relative specific activity of mRNA	Fraction bound to oligo(dT)	Poly A in—	
				Total mRNA	Bound mRNA
Control	—	1.00	70%	10.9%	15.7%
			% of control		
Mechlorethamine	1.0	0.95	103	103	102
CP	60	1.18	80	85	95
BCNU	30	0.80	82	87	96

*Experimental conditions as in table 1; polysomes prepared from same cells as used for preparation of Hn RNA.



——— = Control
—●— = 0.6 mg/kg mechlorethamine
---------- = 1.0 mg/kg mechlorethamine

FIGURE 1.—Sucrose gradient fractionation (5%-25%) from Ehrlich cells treated in vivo with mechlorethamine for 30 mins and then pulsed with ^{3}H-cytidine for 25 mins in the presence of 0.04 μg/ml of actinomycin D. Hn RNA isolated as described in Materials and Methods and centrifuged with 10 mM tris, 0.1 M NaCl, and 2 mM EDTA for 5 hrs at 115,000 × g. ↓18, 28, 45S



——— = Control
—●— = 0.6 mg/kg mechlorethamine
---------- = 1.0 mg/kg mechlorethamine

FIGURE 2.—Sucrose gradient fractionation (5%-25%) from Ehrlich cells treated in vivo with mechlorethamine and then pulsed with ^{3}H-cytidine in the presence of 0.04 μg/ml of actinomycin D. mRNA prepared as described in Materials and Methods and centrifuged as in fig 1 (except for 8 hrs). ↓18, 28S

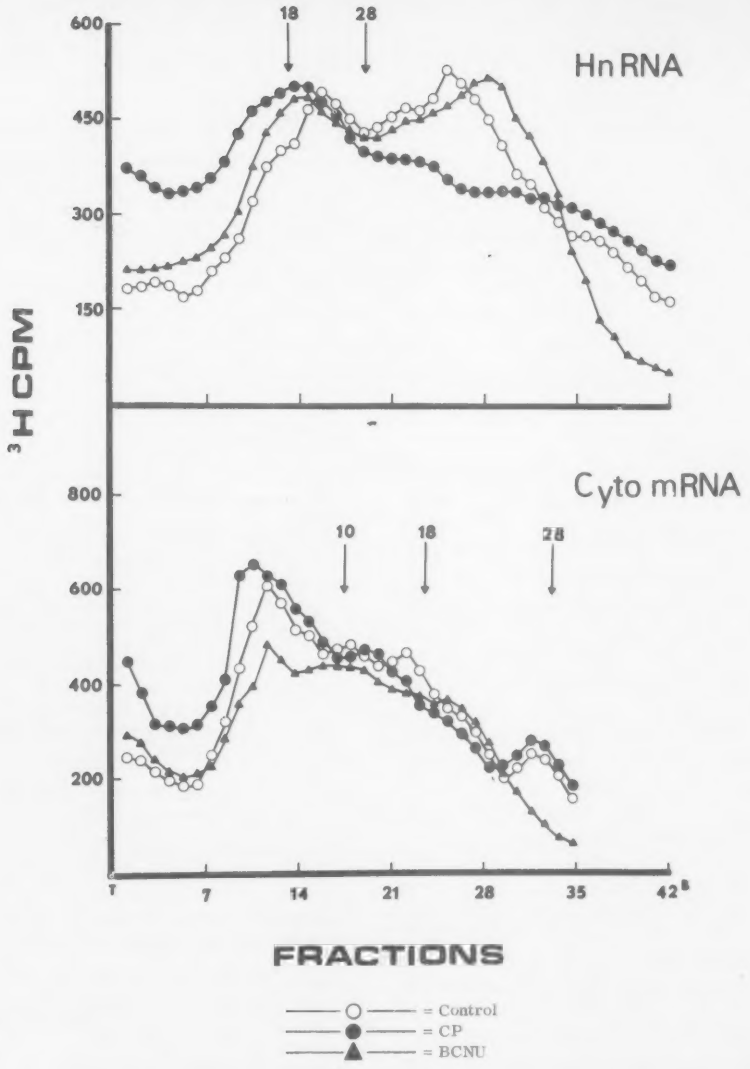


FIGURE 3.—Sucrose gradient fractionation (5%-25%) from Ehrlich cells treated in vivo with CP (60 mg/kg) or BCNU (30 mg/kg). (Same conditions as in figs 1 and 2.)

Evidence from unpublished studies in this laboratory indicates that monofunctional alkylations at the guanine N_7-position are not likely to be the cause of the disturbance in transcription. ^{3}H-diethyl-β-chloroethylamine has been shown to be about 25% more effective in alkylating DNA in vivo compared to mechlorethamine.[4] We have shown that at 6 mg/kg, this agent causes changes in transcription approximately equivalent to those resulting from 0.2-0.4 mg/kg of mechlorethamine; furthermore, the disturbances are rapidly reversible. Thus, the monofunctional agent would require 30 times as many N_7-guaninyl alkylations to produce equivalent effects (12).[4] This is in accordance with the relative lethality of mechlorethamine and its monofunctional analogs and also with the general observation that N_7-guaninyl alkylations are

[4]Taylor B. Synthesis of highly radioactive nitrogen mustard and in vivo alkylation of DNA in mouse tumor cells. PhD dissertation, University of Pennsylvania, Philadelphia, 1973.

TABLE 3.—Preliminary comparison of the effects of alkylating chemotherapeutic agents on transcription in Ehrlich cells*

	Effect of—		
	Mechlorethamine	CP	BCNU
RNA synthesis	0	+	−
Poly A formation	0	−	−
Hn RNA			
$>10^4$ nucleotides	−	0 to −	0
$<2 \times 10^3$ nucleotides	+	+	0
mRNA			
$>3 \times 10^3$ nucleotides	−	−	0
$<10^3$ nucleotides	+	+	0

* 0 = no effect; − = inhibitory effect; + = stimulatory effect.

less active biologically (2).

The frequency of N_7-bis diguaninyl alkylations is roughly 20% of the total while all others comprise 10% (12). If bis alkylations are to terminate transcriptions, on a random-hit basis the target would have to be $> 10^5$ nucleotides and the average size of the premature transcript would still be approximately 5×10^4 deoxynucleotides. This is obviously not the case. The alkylation of 3′ growing RNA termini is even less likely statistically unless these are extremely reactive hot spots. Furthermore, the absence of apparent disturbances in processing after mechlorethamine alkylation makes random termination unlikely unless polyadenylation is under little or no physiologic control or specificity restriction.

It would be tempting to argue that the transcription data reflect specificities in chemical attack, whereby a part of the β-chloroethyl alkylating activity was somehow directed to nuclear components which control the sites of transcription. Since this would undoubtedly be a very subtle interaction, differences in chemical structure could have decisive effects. In any event, certain of these questions may be resolved by current studies, which will involve comparisons with other direct-acting alkylating agents such as methyl methane sulfonate and melphalan (L-phenylalanine mustard).

REFERENCES

1. HIRSCHBERG E, and OCHOA S. Alkylating agents. *In* Experimental Chemotherapy (Schnitzer RJ, and Hawkins P, eds). New York, Academic Press, 1967, vol 5, pp 1-132.
2. SINGER B. The chemical effects of nucleic acid alkylation and relation to mutagenesis and carcinogenesis. Prog Nucleic Acid Res Mol Biol 15:219-284, 1975.
3. ABELSON HT, KARLAN D, and PENMAN S. A comparison of the effects of alkylating agents 1,3-bis(2-chloroethy1)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea and nitrogen mustard on nuclear RNA synthesis and processing. Biochim Biophys Acta 349:389-401, 1974.
4. KANN HE, JR, and KOHN KW. Effects of deoxyribonucleic acid-reactive drugs on ribonucleic acid synthesis in leukemia L1210 cells. Mol Pharmacol 8:551-560, 1972.
5. PENMAN S, SCHERRER K, BECKER Y, ET AL. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger RNA. Proc Natl Acad Sci USA 49:654-662, 1963.
6. WETTSTEIN FO, STACHLIN T, and NOLL H. Ribosomal aggregate engaged in protein synthesis; characterization of the ergosome. Nature 197:430-435, 1963.
7. AVADHANI NG, BATTULA N, and RUTMAN RJ. Messenger ribonucleic acid metabolism in mammalian mitochondria. Origin of ethidium bromide resistant poly(adenylic acid) containing ribonucleic acid in Ehrlich ascites mitochondria. Biochemistry 12:4122-4129, 1973.
8. AVADHANI NG, LEWIS FS, and RUTMAN RJ. Messenger RNA metabolism in mammalian mitochondria. Quantitative aspects of structural information coded by the mammalian genome. Biochemistry 13:4638-4645, 1974.
9. AVIV H, and LEDER P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 69:1408-1412, 1972.
10. DAVIDSON EH, and BRITTEN RJ. Organization, transcription, and regulation in the animal genome. Q Rev Biol 48:565-613, 1973.
11. HUTCHISON DJ. Cross resistance and collateral sensitivity studies in cancer chemotherapy. Adv Cancer Res 7:235-350, 1963.
12. CHUN EHL, GONZALES L, LEWIS FS, ET AL. Differences in the in vivo alkylation and cross-linking of nitrogen mustard-sensitive and -resistant lines of Lettré-Ehrlich ascites tumors. Cancer Res 29:1184-1194, 1969.
13. LAWLEY PD. Effects of some chemical mutagens and carcinogens on nucleic acids. Prog Nucleic Acid Res Mol Biol 5:89-131, 1966.

Aspects of the Teratology of Cyclophosphamide (NSC-26271)[1]

R. Ashby,* L. Davis, B. B. Dewhurst, R. Espinal, R. N. Penn, and D. G. Upshall[2]

SUMMARY

Wistar-derived rat embryos were most sensitive to maternally administered cyclophosphamide on Days 9.5 and 10.5 of gestation and to chlorambucil on Day 9.5 of gestation. The spectrum of malformations and the dose-response behavior of both drugs were similar. Chlorambucil was three times more potent than cyclophosphamide on an mg/kg basis. The drugs showed additive behavior when administered in combination. The teratogenic effects of the individual or combined drugs were reduced by protective doses of cysteine or glutathione. Studies with analogs of cyclophosphamide have suggested that the whole molecule is involved in teratogenesis.

[Cancer Treat Rep 60:477-482, 1976]

Mechlorethamine is a potent teratogen in the rat (1,2). Cyclophosphamide (CP) possesses teratogenic activity in chicks (3), mice (4), rats (5), and rabbits (6) and has been implicated in human teratogenesis (7,8). Despite the similarity between mechlorethamine- and CP-induced malformations (9), the presence of unchanged CP in the embryo is apparently required for teratogenesis (10-12). The mechanism of teratogenesis is not understood although the alkylation of embryonic macromolecules (13), which may be DNA (14) or mRNA (15), has been reported. We have investigated the teratology of CP by comparing its teratogenic characteristics with those of structural analogs and another teratogen, the alkylating agent chlorambucil (CH) (16-18).

MATERIALS AND METHODS

Primiparous Wistar-derived rats were used. Midday on the day that vaginal smears were positive was termed Day 0.5 of gestation. The rats were injected on single days and killed on Day 20.5 of pregnancy. The uterine contents were examined for live and dead fetuses and resorption sites. Live fetuses were weighed (±50 mg) and examined for malformation by skeletal staining (19) or internal visceral observation (20). CP, cysteine, free base (Sigma Chemical Co), and glutathione (Sigma Chemical Co) were dissolved in isotonic saline, neutralized, and administered ip. CH was suspended in a 2.5% vol/vol aqueous solution of absolute ethanol and was given orally. Control groups received the respective vehicles. When administered in combination the alkylating agents were injected simultaneously. Cysteine and glutathione were injected 30 minutes before the alkylating agents. Analogs of CP (prepared by R. N. Penn) were suspended in 0.1% Tween 80 in isotonic saline and administered ip to Charles River mice.

RESULTS

The period of rat embryogenic sensitivity to CP was investigated by administering 10 mg/kg on single days of gestation. Maximum response, manifested by embryolethality, was observed on Days 9.5 and 10.5 (fig 1). Earlier or later administrations during organogenesis produced malformations. No significant numbers of malformations were found after treatments during the preimplantation stage (Days 0.5-6.5) or during the fetal growth stage (Days 14.5-20.5).

Doses of 10 mg/kg given on Days 7.5 and 8.5, and doses of 8 mg/kg given on Day 9.5 produced a marked increase in the incidence of microphthalmia (spontaneous in the colony at a per litter index[3] of 0.04) and internal hydrocephalus. Later

[1]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate .

[2]School of Natural Sciences, The Hatfield Polytechnic, Hertfordshire, England.

*Reprint requests to: Dr. R. Ashby, Life Science Research, Stock, Essex, CM4 9PE, England.

[3]*Per litter index* is the mean fraction of implants in each litter with the scored characteristic.

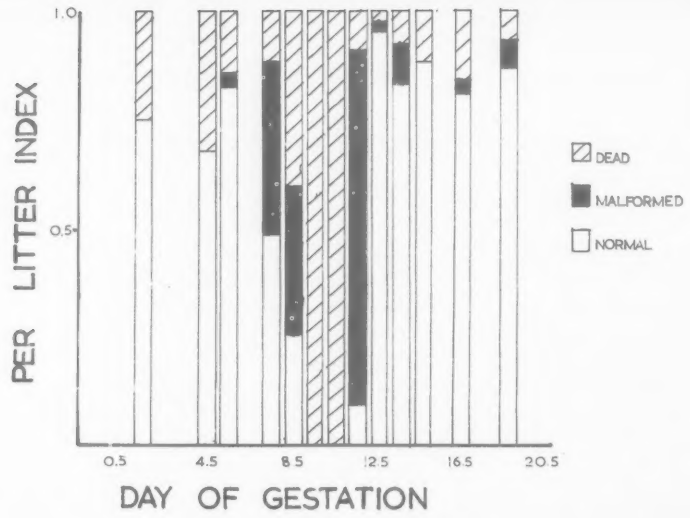


FIGURE 1.—CP period of susceptibility.

administrations produced internal hydrocephalus and subcutaneous edema. Few skeletal defects were noted.

The ip administration of CP to Charles River mice at doses of 10-20 mg/kg on Day 9.5 produced a wide variety of ophthalmic and skeletal malformations.

Rat embryonic sensitivity to CH, demonstrated by malformation (3 mg/kg) or embryolethality (6 mg/kg), was greatest on Day 9.5 of gestation (fig 2). Few malformations were noted after treatment with 3 mg/kg on subsequent days of pregnancy but a wide range of malformations were observed at 6 mg/kg. Microphthalmia and internal hydrocephalus

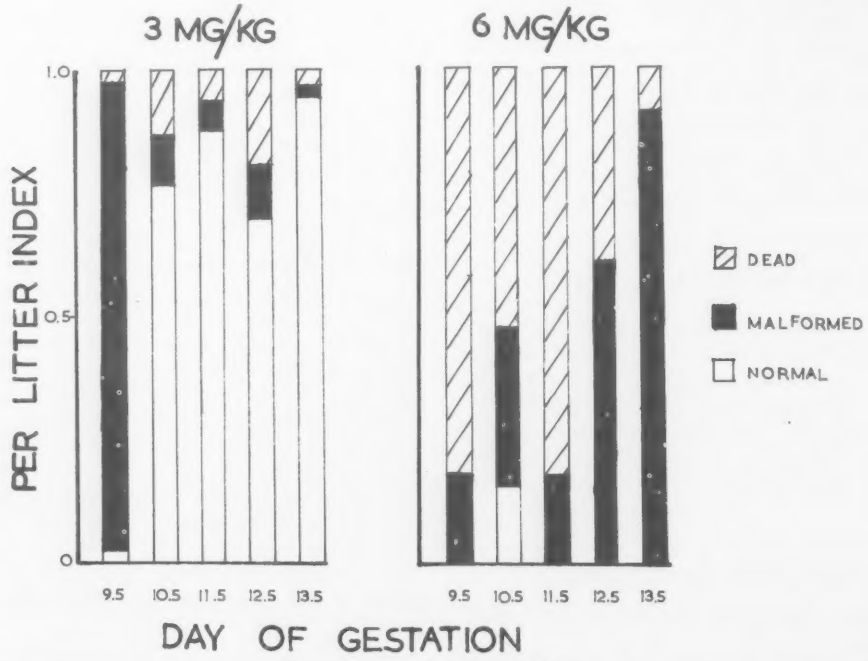


FIGURE 2.—CH period of susceptibility.

were produced by treatments given on Days 9.5 and 10.5. Peak periods of sensitivity were observed for the caudal vertebrae (Days 11.5 and 12.5), forelimbs (Day 12.5), and hindlimbs and cranium (Day 13.5). The teratogenic potency of CH was not reduced by oral dosing when compared with the results obtained by ip dosing (2,17,21,22).

The similarity in teratogenic characteristics of CP and CH prompted a comparison of their dose-response behavior using the Day-13.5 rat embryo as the model. Dose-related teratogenic activity, both in terms of the number of affected fetuses and the severity of malformations, was observed with both drugs (table 1). The incidence of malformation increased above points of inflection at 10 mg/kg (CP) and 3 mg/kg (CH). Doses above these levels elicited greater response with respect to the spectrum of malformation and fetal death (which was probably a consequence of extreme malformation). Predominant among the malformations were subcutaneous edema and suprascapular hematoma. A comparison of the dose-response curves (fig 3) shows that both drugs produced a sigmoidal dose-effect relationship.

A kinetic analysis (23) of these results demonstrated the greater potency of CH (three times greater on an mg/kg basis) and also showed that both drugs conformed to class 2 teratogen behavior, implying the requirement for the saturation of sets of receptors before the production of teratogenesis (fig 4).

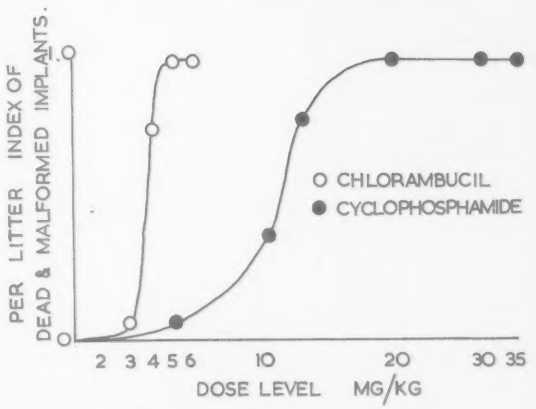


FIGURE 3.—Dose-response curves: Day 13.5.

The similarities in dose-response behavior were consolidated by a potency probit analysis (table 2).

TABLE 1.—Effects of variation of dose level of CP and CH on the Day-13.5 rat embryo

Drug	Dose level (mg/kg)	No. of implants	% of animals		
			Normal	Malformed	Dead
CP	0	140	89.6	1.4	10
	5	69	93	1	6
	10	56	63	9	28
	12.5	55	22	62	16
	20	37	0	87	13
	30	18	0	100	0
	35	26	0	19	81
CH	0	91	91	0	9
	3	60	96	2	2
	4	36	25	69	6
	5	44	0	91	9
	6	52	0	92	8
	12	65	0	72	28

TABLE 2.—Potency probit analysis for CP and CH

Statistical unit	Mother		Implant	
	CP	CH	CP	CH
TD30	10.1	3.37	10.7	3.43
TD50	10.9	3.66	11.4	3.65
TD90	13.4	4.47	13.4	4.27
Probit lines	Parallel		Parallel	
Potency ratio	1	2.99	1	3.12

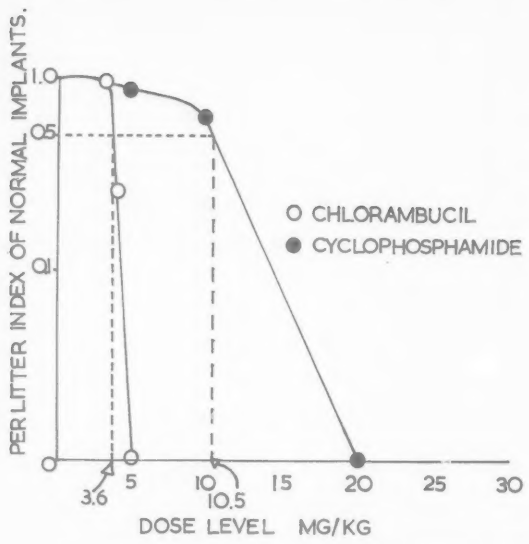


FIGURE 4.—Kinetic analysis of Day-13.5 dose response.

The analysis demonstrated that regardless of the statistical unit chosen (mother or implantation) the probit lines of CP and CH were parallel and the potency ratio on an mg/kg basis was approximately 1:3 (CP:CH). This information was used to compare the kinetic behavior of the drugs. Potency correction resulted in superimposed curves (fig 5). Further evidence for the similar nature of the teratogens was obtained by a comparison of the spectrum of abnormalities produced by equivalent doses (table 3).

TABLE 3.—Spectrum of anomalies produced by equipotent doses of CP and CH

% of animals—	CP (12.5 mg/kg)	CH (4 mg/kg)
Normal	22	25
Dead	16	6
Malformed	62	69
With cranial defects	48	22
With rib defects	2	9
With forelimb defects	11	19
With hindlimb defects	67	62
Mean fetal weight (g)	2.69	2.77

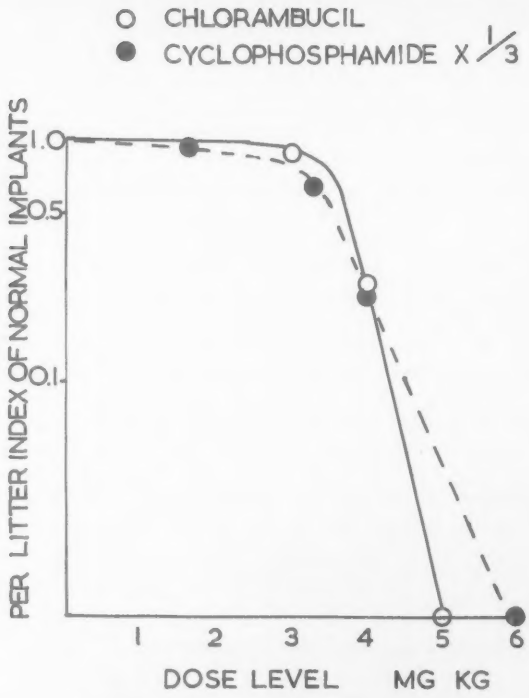


FIGURE 5.—Potency corrected; kinetic analysis.

The relationship between the two drugs was also tested by their administration in combined doses. We chose to use various dose levels which were all individually at or below the apparent thresholds for teratogenicity but which in combination represented a range in equivalent dose level of CH between 3.16 and 6.66 mg/kg. The total effects of these treatments showed an apparent threshold dose of 3.4 mg/kg equivalents of CH. Maximum response was achieved at 4.83 mg/kg equivalents of CH. Kinetic analysis of the results showed class 2 teratogen behavior that superimposed on the matched dose-response curves of the individual drugs (fig 6), indicating an additive effect between the agents in combination.

Protection against teratogenic doses of CP, alone or in combination with CH, was afforded by cysteine and glutathione (table 4). Significant reduction ($P < 0.05$) in the incidence of malformation provoked by 12.5 mg/kg of CP was achieved with cysteine at a dose of 150 mg/kg. Glutathione, at dose levels of 380 or 570 mg/kg, was protective against CP at 12.5 mg/kg or a combination of CP (10 mg/kg) and CH (3 mg/kg).

Further studies to assess the importance of an alkylating moiety in the teratogenicity of CP em-

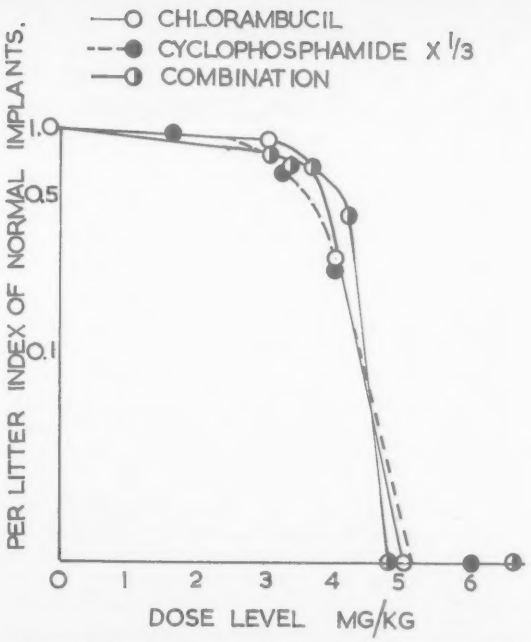


FIGURE 6.—Comparison of individual and combined treatments.

ployed a number of structural analogs (fig 7) which were designed to include or exclude the alkylating potential of the mustard portion and to modify the lability of the substituted amino group by using different heteroatoms in the cyclic system. All analogs exhibited similar general toxicity in mice (estimated by the LD50) but none was teratogenic at doses four times the teratogenic dose of CP.

DISCUSSION

The observation that inhibition of mixed-function oxidase activity resulted in enhanced teratogenicity of CP led to the proposition that the whole molecule was responsible for teratogenesis

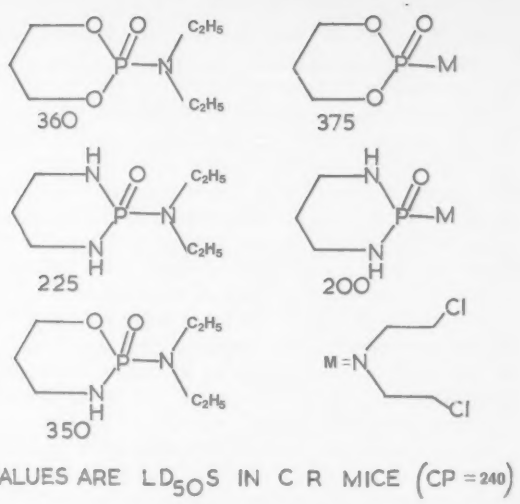


FIGURE 7.—LD50s of CP analogs.

(4). However, CP is not an alkylating agent per se (24) and the embryo does not possess the microsomal enzymes necessary for its activation (25), although the alkylation of embryonic macromolecules has been reported (13-15).

There are a number of possible teratogenic mechanisms. Firstly, the whole molecule may be slowly degraded, either spontaneously or enzymatically, within the embryo to produce teratogenic alkylating products. This seems unlikely since the teratogenic characteristics of CP are those of a rapidly acting agent. It is also possible that the whole molecule enters the embryo and acts teratogenically without further breakdown, perhaps by the inhibition of enzyme-mediated differentiating processes. Alternatively, the entry of the whole molecule into the embryo may be coincidental with the entry of effective alkylating maternal metabolites or with the placental metabolism of the drug.

TABLE 4.—Protective effects of cysteine and glutathione against the teratogenic activity of CP and CH

Dose (mg/kg) of—				No. of dams	Per litter index		Mean fetal weight (g)
CP	CH	Cysteine	Glutathione		Dead	Live, malformed	
12.5				6	0.19	0.72	2.68
12.5		150		6	0.12	0.27*	2.83
12.5		150		6	0.12	0.07*	2.80
12.5			380	4	0.2	0*	2.65
5.0	3.0			9	0.15	0.52	2.66
5.0	3.0		418	6	0.11	0*	2.81
10.0	3.0			9	0.17	1.0	2.22
10.0	3.0		570	6	0.3	0.28*	2.50

*Significantly different ($P = 0.05$) from unprotected group.

Finally, maternal homeostasis or maternofetal exchange may be affected by the whole drug or its metabolites.

The evidence presented here suggests that the drug is a potent teratogen exerting a rapid effect. Its teratogenic characteristics resemble those of the alkylating agent CH. The study with combined doses of the two drugs indicates that their teratogenic modes of action are similar in that the effects are additive and the slopes of the dose-response curves are the same. Alkylating activity is associated with this action as demonstrated by the protection afforded by cysteine and glutathione, although the biologic activity of the analogs suggests that the whole molecule of CP has a special function in teratogenesis.

It is of interest that mechlorethamine is most potent as a teratogen when denied access to the embryo, implying a major effect on the dam (26). The kinetic behavior of CP and CH is that of the class 2 teratogens which may mediate their effects through the mother (23). The production of subcutaneous edema and localized hematomas in this and other studies (27) resembles the "edema syndrome" (28) which may thus be involved in teratogenesis. This syndrome has been produced by maternal hypoxia (29) and by agents acting on the nutritional process of the early rat embryo (30).

It appears that a special property of the whole molecule, in combination with its alkylating potential, is involved in teratogenesis. Despite the well-established entry of the drug into the embryo (11), the possible effects of the agent on the mother and the maternofetal exchange should not be disregarded.

REFERENCES

1. Haskin D. Some effects of nitrogen mustard on the development of the external body form in the fetal rat. Anat Rec 102:493-511, 1948.
2. Murphy ML. A comparison of the teratogenic effects of five polyfunctional alkylating agents on the rat fetus. Pediatrics 23:231-244, 1959.
3. Singh S, Tuli SM, and Gupta PK. Skeletal defects induced by cyclophosphamide (Endoxan-Asta) in chick embryos—a preliminary report. Acta Orthop Scand 42:217-226, 1971.
4. Shoji R, and Ohzo E. Some aspects of the effect of Endoxan on developing mouse embryos. J Fac Sci Hokkaido Zool 15:662-665, 1965.
5. Chaube S, Kury G, and Murphy ML. Teratogenic effects of cyclophosphamide (NSC-26271) in the rat. Cancer Chemother Rep 51:363-379, 1967.
6. Clavert A. Various types of ocular anomalies induced in rabbit embryos by injection of cyclophosphamide into pregnant females. C R Soc Biol (Paris) 164:1344-1346, 1970.
7. Greenberg LH, and Tanaka KR. Congenital anomalies probably induced by cyclophosphamide. JAMA 188:423-426, 1964.
8. Toledo TM, Harper RC, and Moser RH. Fetal effects during cyclophosphamide and irradiation therapy. Ann Intern Med 74:87-91, 1971.
9. Gibson JE, and Becker BA. The teratogenicity of cyclophosphamide in mice. Cancer Res 28:475-480, 1968.
10. Gibson JE, and Becker BA. Effect of phenobarbital and SKF 525A on the teratogenicity of cyclophosphamide in mice. Teratology 1:393-398, 1968.
11. Gibson JE, and Becker BA. Effect of phenobarbital and SKF 525A on placental transfer of cyclophosphamide in mice. J Pharmacol Exp Ther 177:256-262, 1971.
12. Gibson JE, and Becker BA. Teratogenicity of structural truncates of cyclophosphamide in mice. Teratology 4:141-150, 1971.
13. Short RD, and Gibson JE. ^{14}C-cyclophosphamide alkylation of mouse embryo macromolecules. Proc Soc Exp Biol Med 145:620-624, 1974.
14. Murthy VV, Becker BA, and Steele WJ. Effects of dosage, phenobarbital, and 2-diethylaminoethyl-2,2-diphenylvalerate on the binding of cyclophosphamide and/or its metabolites to the DNA, RNA, and protein of the embryo and liver in pregnant mice. Cancer Res 33:664-670, 1973.
15. Kohler E, and Merker HJ. Effect of cyclophosphamide pretreatment of pregnant animals on the activity of nuclear DNA-dependent RNA polymerases in different parts of rat embryos. Naunyn Schmiedebergs Arch Pharmacol 277:71-88, 1973.
16. Monie IW. Chlorambucil induced abnormalities of the urinogenital system of rat fetuses. Anat Rec 139:145-153, 1961.
17. Murphy ML, Del Moro A, and Lacon C. The comparative effects of five polyfunctional alkylating agents on the rat fetus with additional notes on the chick embryo. Ann NY Acad Sci 68:762-781, 1958.
18. Shotton D, and Monie IW. Possible teratogenic effect of chlorambucil on a human fetus. JAMA 186:74-75, 1963.
19. Williams TW. Alizarin red S and toluidine blue for differentiating adult or embryonic bone and cartilage. Stain Technol 16:23-25, 1941.
20. Wilson JG. Methods for administering agents and detecting malformations in experimental animals. *In* Teratology. Principles and Techniques (Wilson JG, and Warkany J, eds). Chicago, The University of Chicago Press, 1965, pp 262-277.
21. Murphy ML. Teratogenic effects of tumour inhibiting chemicals. *In* CIBA Foundation Symposium on Congenital Malformations (Wolstenholme GEW, and O'Connor CM, eds). London, Churchill, 1960, pp 78-107.
22. Kernis MM. Cysteine interference with chlorambucil-induced teratogenesis in the rat. Proc Soc Exp Biol Med 139:62-65, 1972.
23. Jusko WJ. Pharmacodynamic principles in chemical teratology: dose-effect relationships. J Pharmacol Exp Ther 183:469-480, 1972.
24. Foley GE, Friedman OM, and Drolet BP. Studies on the mechanism of action of Cytoxan. Evidence of activation in vivo and in vitro. Cancer Res 21:57-63, 1961.
25. Short RD, and Gibson JE. The relationship of cyclophosphamide activation to its perinatal toxicity in mice. (Abstr 91) Toxicol Appl Pharmacol 19:397, 1971.
26. Brent RL, Bolden BT, and Weiss A. The use of uterine vascular clamping in the pregnant rat to modify the embryotoxic effects of anticancer drugs. Cancer Res 28:2001-2006, 1968.
27. Singh S. The teratogenicity of cyclophosphamide (Endoxan-Asta) in rats. Indian J Med Res 59:1128-1135, 1971.
28. Grabowski CT, and Schroeder RE. A time-lapse photographic study of chick embryos exposed to teratogenic doses of hypoxia. J Embryol Exp Morphol 19:347-362, 1968.
29. Petter C, Bourbon J, Maltier J-P, et al. Production d'hémorragies des extrémités chez le foetus de rat souris à une hypoxie in utero. C R Acad Sci [D] [Paris] 272:2488-2490, 1971.
30. Waddington CH, and Carter TC. A note on abnormalities induced in mouse embryos by trypan blue. J Embryol Exp Morphol 1:167-180, 1953.

The Use of Deuterated Analogs in Qualitative and Quantitative Investigations of the Metabolism of Cyclophosphamide (NSC-26271)[1,2]

P. J. Cox,[3,4] P. B. Farmer,[3] A. B. Foster,[3] E. D. Gilby,[5] and M. Jarman[5,6,*]

SUMMARY

Ring-deuterated analogs of cyclophosphamide (CP) (4-d_2, 5-d_2, 4,6-d_4, and 4,5,6-d_6 derivatives) have been used to study the influence of deuterium substitution on the rates of metabolic pathways involving oxidation at C-4, and on the rate of elimination of acrolein from aldophosphamide. The magnitude of the deuterium isotope effect (k_H/k_D) associated with appropriate C-deuteration has been related to antitumor activity against the ADJ/PC6 murine plasma cell tumor. Isotope effects of 2.2 and 1.8 respectively, for the formation of 4-ketocyclophosphamide (4-keto-CP) and carboxyphosphamide, caused little or no change in antitumor activity (4-d_2 and 4,6-d_4 analogs compared with CP), but an isotope effect of about 5.3 for the β-elimination pathway, consequent on 5,5-dideuteration, was paralleled by a marked drop in potency (7-13-fold increase in ED90) of 5,5-dideuterated analogs compared with that of CP. Analogs tetradeuterated in the bis(2-chloroethyl)amino function were used to quantitate CP and 4-keto-CP in human plasma and urine using stable-isotope dilution and direct-insertion electron impact mass spectrometry. The negative optical rotation of CP recovered from human urine after administration of the racemic drug gave evidence for stereoselectivity in the metabolism.

[Cancer Treat Rep 60:483-491, 1976]

Analogs of drugs and metabolites in which one or more atoms have been replaced by a stable isotope have been utilized in several areas of drug research (1). Thus, following the biodegradation of a drug analog, the presence of the isotope in metabolites can often be determined by physical methods, eg, nuclear magnetic resonance spectroscopy and mass spectrometry. Deuterated analogs can often conveniently replace radioactively labeled drugs in qualitative and quantitative investigations of metabolism, particularly in man where the radioactivity presents an ethical problem.

As part of our investigations into the metabolism of cyclophosphamide (CP), we have utilized stable-isotope labeling (specifically, deuterium labeling) in two main areas.

Deuterium isotope effects.—Several of the metabolic steps shown in figure 1 involve oxidative cleavage of a C-H bond, and there could be an isotope effect consequent on a slower rate for C-D cleavage in an appropriate C-deuterated analog (2). We have therefore systematically studied the influence of deuterium substituents on the metabolism and antitumor activity of CP with the aim of defining the relative importance of the various metabolic pathways (fig 1) as determinants of antitumor activity and drug toxicity. There is also the intriguing possibility of altering the biologic activity of CP by specific deuterium labeling as has been observed in other areas of drug research (3).

[1]Supported by grants G973/786-7/K from the Medical Research Council; the AEI-MS12 mass spectrometer was purchased on a special grant (G969/189/C).

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England.

[4]Ward Blenkinsop Fellow.

[5]Unit of Human Cancer Biology, London Branch, Ludwig Institute for Cancer Research, in conjunction with the Royal Marsden Hospital, London, England.

[6]We gratefully acknowledge contributions to this study from Mr. M. H. Baker, Dr. P. Ciclitera, Mr. L. J. Griggs, and Mr. M. Jones.

Reprint requests to: Dr. M. Jarman, Institute of Cancer Research, Clifton Ave, Sutton, Surrey, SM2 5PX, England.

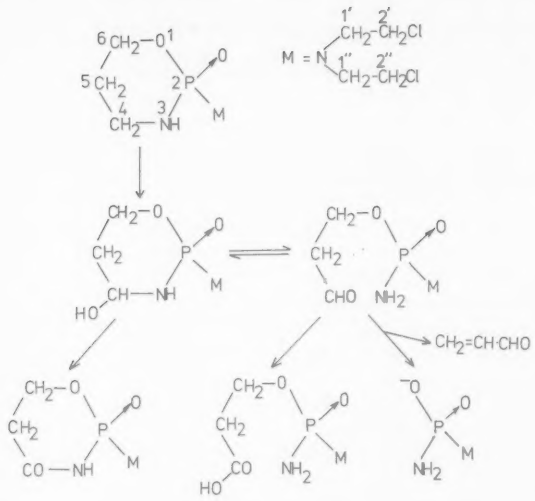


FIGURE 1.—Scheme for the metabolism of CP (7).

Quantitation by mass spectrometry/stable-isotope dilution.—In this procedure (4) known quantities of deuterated analogs are added to a biologic fluid (eg, blood or urine) and the levels of the drug and its metabolites are determined after suitable isolation procedures from the ratio of intensities of corresponding appropriate signals in the mass spectrum of each pair of protium and deuterium forms.

MATERIALS AND METHODS

Isolation and Identification of CP and its Metabolites

Thin-layer chromatography (TLC) was conducted on glass plates (20 × 20 or 20 × 5 cm) coated with silicic acid (Merck Kieselgel G). Radioactive components were detected with a Berthold radiochromatogram scanner. 2,4-Dinitrophenylhydrazones were detected visually (yellow or orange bands). For column chromatography on silicic acid, Merck Kieselgel 60 was used. Mass spectra were determined with an AEI-MS12 spectrometer using the direct-insertion technique, an ionizing voltage of 70 eV, and a source temperature of about 100°C.

Measurement of Antitumor Activities

The effectiveness of CP and its deuterated analogs against the advanced ADJ/PC6 tumor in female BALB/c mice (table 1) was tested by previously described methods (5).

Quantitative Analysis

The synthesis of deuterated standards is reported elsewhere (6). Blood and urine samples were taken from a woman (patient No. 1, wt 55 kg) with oat cell carcinoma of the lung, who had received CP (1 g) iv as part of a previously reported protocol (4).

The method for obtaining ratios between relevant peak heights in the mass spectra of samples, and for the validation of the ratio measurements using standard mixtures of drug or metabolite with deuterated analog, were those previously reported (4).

Synthesis of Ring-Deuterated Analogs: CP-4,5,6-d_6 and CP-5-d_2

a. 1-Amino-1,1,2,2,3,3-hexadeuteropropan-3-ol.—A mixture of ethyl cyanoacetate (2 g) and D_2O (20 ml) was heated under reflux for 5 minutes. The solution was cooled and extracted with benzene (2 × 20 ml), and the dried ($MgSO_4$) extract was concentrated. The product (oil, 1.8 g) was subjected twice more to the exchange procedure. Final recovery was 1.2 g. A solution of $LiAlD_4$ (1 g stirred with dry ether [30 ml] for 30 mins and filtered [glass sinter]) was added to a solution of this product (1 g) in dry ether (10 ml) at 0°C. The mixture was

TABLE 1.—Activity of CP and deuterated analogs against the mouse plasma cell tumor ADJ/PC6

Compound	ED90 (mg/kg)	LD50 (mg/kg)	Therapeutic index (LD50/ED90)
CP*	3.6	335	93
CP-4-d_2*	3.7	360	97
CP-5-d_2†	47.5	>500	>10.5
CP-4,6-d_4*	7.6	305	40
CP-1′, 1″-d_4†	4.8	220	45.8
CP-4,5,6-d_6*	26	>640	>24.6

*Twofold dose spacing.
†Fivefold dose spacing.

heated under reflux for 16 hours, then cooled and treated with saturated aqueous Na_2SO_4 (5 ml). Insoluble material was collected and extracted (Soxhlet) with ether for 2 days. The combined filtrate and extracts were concentrated to a yellow oil (0.34 g) which was used without further purification for the next stage. Mass spectral data were as follows: m/e 81 [1% of base peak at m/e 32, $M^{+\cdot}$ for $HO(CD_2)_3NH_2$].

b. CP-d_6.—*N,N*-bis(2-chloroethyl)phosphoramidic dichloride (1 g) was added to a stirred mixture of the product from (*a*) and 1,2-dichloroethane (5 ml) containing triethylamine (0.5 ml). After 2 hours at room temperature, water (5 ml) was added and the mixture was extracted with dichloromethane (20 ml). The dried (Na_2SO_4) extract was concentrated and applied to a column (25 × 2 cm) of silicic acid (30 g) which was eluted with chloroform (10-ml fractions). The required product (fractions 60-120) crystallized from water as colorless plates (0.135 g) had a melting point of 40°C-41°C. The ratio m/e 217:216 ($[M\text{-}CH_2Cl]^+$ for the d_6 and the d_5 analog respectively) showed that the product contained the d_6 and the d_5 analogs in the ratio of 91:9.

c. CP-d_2.—Ethyl cyanodideuteroacetate, prepared by the above exchange procedure, was reduced with $LiAlH_4$ as described in (*a*) to yield 1-amino-2,2-dideuteropropan-3-ol, $M^{+\cdot}$ at m/e 77. A procedure parallel to that described in (*b*) was then used to obtain CP-5-d_2 as colorless plates (0.24 g), melting point 42°C-44°C. Major signals in the mass spectrum are shown in table 2. From the ratio m/e 213:212 ($[M\text{-}CH_2Cl]^+$ for the d_2 and the d_1 analog respectively) the product contained the d_2 and the d_1 analogs in the ratio of 93:7.

Metabolism

The conditions for obtaining rat-liver microsomes and 9000 g_{av} supernatant were those previously reported (7) as was the composition of incubation media unless otherwise stated. Details of the added substrates are given below.

a. Metabolism of a mixture of CP and its 4-d_2, 4,6-d_4, and 4,5,6-d_6 analogs with rat-liver microsomes.—The incubation mixture (60 ml) contained about 3 mg of each substrate and a trace of 5-^{14}C-CP (New England Nuclear, Swindon, England). A 2-ml aliquot of the incubation mixture was immediately treated with 4 volumes of redistilled ethanol to precipitate protein. This procedure was repeated after 20 minutes on a second aliquot. After centrifugation (1200 g_{av}) and concentration of each supernatant, the residue was extracted with chloroform and the concentrated extract was subjected to mass spectrometry without further purification. Signal:background ratios for the appropriate CP peaks ($[M\text{-}N(CH_2CH_2Cl)_2]^+$ and $[M\text{-}CH_2Cl]^+$; for m/e values *see* table 2) were >100:1. CP consumption (table 3) was measured by a previously described procedure based on the measurement of radioactively labeled drug (8). The ratios between the

TABLE 2.—M/e values for structurally equivalent ions in the mass spectra of CP and deuterated analogs

Compound	$M^{+\cdot}$	$[M\text{-}CH_2Cl]^+$	$[M\text{-}N(CR_2CH_2Cl)_2]^+$ (R = H or D)	$[ClCH_2CR_2NH{=}CR_2]^+$ (R = H or D)
CP	260	211	120	92
CP-4-d_2	262	213	122	92
CP-5-d_2	262	213	122	92
CP-4,6-d_4	264	215	124	92
CP-1′,1″-d_4	264	215	120	96
CP-4,5,6-d_6	266	217	126	92

TABLE 3.—Relative extents of metabolism by rat-liver microsomes after incubation for 20 mins of a mixture of CP and ring-deuterated analogs

Compound	Ratio of deuterated analog:CP (CP = 100)		% metabolized
	Initial mixture	After 20-min incubation	
CP	100	100	76
CP-4-d_2	85	96	72
CP-4,6-d_4	79	96	69
CP-4,5,6-d_6	62	81	66

components, and the extent of metabolism of each, are shown in table 3.

b. Metabolism of a mixture of CP and its 4,6-d_4 analog by rat-liver 9000 g_{av} supernatant.—The incubation mixture (30 ml) contained about 3 mg of each substrate and a trace of 5-^{14}C-CP. Aliquots (2 ml) of the mixture were taken initially and after 30 minutes. Protein was precipitated (N HCl) and each supernatant was passed through a column (8 × 1 cm) of Amberlite XAD-2 resin, which was eluted with water and then with methanol. Methylation (ethereal diazomethane) of the products in the methanol eluates followed by TLC in ethyl acetate partially resolved the mobile components which were identified by mass spectrometry (*see* Results). The ratios of protium:deuterium forms (d_0:d_4 for CP and d_0:d_2 for 4-ketocyclophosphamide [4-keto-CP] and carboxyphosphamide) were determined from the appropriate mass spectra.

c. Metabolism of a mixture of CP and its 5-d_2 analog by rat-liver microsomes.—The conditions for incubation of the mixture of substrates (2.5 mg of each in 30 ml of medium) were as described in (a) except that phosphate buffer (0.15 M) replaced tris-HCl buffer of the same pH (7.4). The CP consumed was assayed by the addition of CP-4,6-d_4 (10 μl of a solution containing 0.93 mg/ml) to aliquots (1 ml) of the incubation mixture, and the ratio of d_0:d_4 species ($[M\text{-}N(CH_2CH_2Cl)_2]^+$ at m/e 120, 124) was measured in the mass spectrum of recovered CP (4).

The ratio of CP:CP-5-d_2 was simultaneously obtained. After 30 minutes, air which had been passed through a saturated solution of 2,4-dinitrophenylhydrazine in N HCl (30 ml) to remove atmospheric contaminants reactive to this reagent, was gently bubbled through the incubation mixture, still maintained at 37°C, and subsequently passed through more 2,4-dinitrophenylhydrazine reagent (2 ml) to trap the released acrolein. The trapping solution was replaced at the specified intervals (table 4). After neutralization (N NaOH), each trapping solution was extracted with benzene and the concentrated extracts were subjected to TLC, using as markers the authentic 2,4-dinitrophenylhydrazones of acetone and acrolein. Acetone was a common artefact in previous experiments in which acrolein was formed from CP metabolites, despite precautions taken to remove it. TLC in diethyl ether-petroleum ether (boiling point 40°C-60°C) (1:1) separated components corresponding in R_f value to the markers (R_f values 0.28 and 0.35 respectively) but failed to separate the acrolein derivative from a component of molecular weight 202 (identified as 2,4-dinitrochlorobenzene); separation was effected by TLC in benzene (R_f values 0.58 and 0.68 respectively). The mass spectrum of the acrolein derivatives gave the ratios between acrolein and acrolein-d collected during the specified time intervals (*see* Results and table 4).

TABLE 4.—Ratios of acrolein:acrolein-d collected during various time intervals after incubation for 30 mins of an equimolar mixture of CP and its 5-d_2 analog with rat-liver microsomes at 37°C

Time (mins)	Ratio of acrolein: acrolein-d
0-10	5.3
10-30	3.0
30-80	2.5
80-160	1.6

Assay of CP and 4-Keto-CP in Plasma

Aliquots (2 ml) of plasma obtained from blood taken at various time intervals (fig 2) after injections of CP were each treated with 20 μl of a standard solution containing CP-d_4 and 4-keto-CP-d_4 (1.00 mg of each) in water (1 ml). The samples were extracted with ethyl acetate (2 ml). The organic phase was decanted and treated with an ethereal solution (1 ml) of diazomethane. After 10 minutes the solution was concentrated and subjected to TLC using ethyl acetate. Silicic acid in the regions corresponding in R_f value to CP (R_f 0.1) and the *N*(3)-methyl derivative of 4-keto-CP (R_f 0.3) was removed and eluted with methanol. The concentrated eluates were subjected to mass spectrometry and the ratios between appropriate signal intensities—for m/e 211, 215 and m/e 239, 243; $([M\text{-}CH_2Cl]^+$ ions for d_0 and d_4 analogs respectively of CP and *N*(3)-methyl-4-keto-CP)—were used for determining the appropriate components. The results are shown in figure 2.

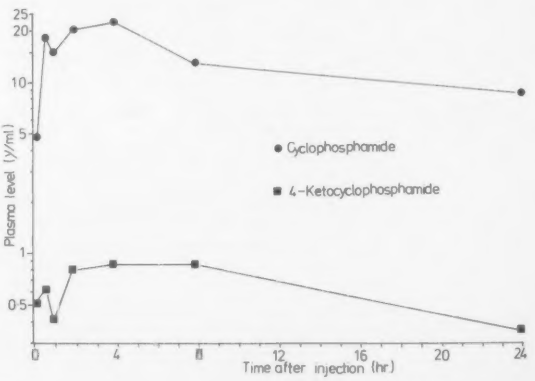


FIGURE 2.—Plasma levels of CP and 4-keto-CP after iv administration of CP (1 g, 18 mg/kg).

Assay of CP and 4-Keto-CP in Urine

Aliquots (1 ml) from urine collected 4 and 24 hours after drug administration were treated with the standard solutions (10 μl of each, see above) and applied to a column (7 × 1 cm) of washed Amberlite XAD-2 resin. The column was eluted with water (25 ml), drained under mild suction, and then eluted with methanol (5 ml). The methanol eluate was concentrated to dryness, and a solution of the residue in methanol (1 ml) was treated with ethereal diazomethane (1 ml). After 10 minutes, the solution was concentrated to a small volume before concentration onto the direct-insertion probe of the mass spectrometer. From the mass spectrum, the concentration of CP and of 4-keto-CP (see Results) was obtained by measuring the ratios m/e 211:215 and m/e 239:243.

Isolation of Optically Active CP From Human Urine

The total 24-hour urine (500 ml) was collected from a woman (patient No. 2) treated with racemic CP (1 g) as described above, and extracted with chloroform (3 × 500 ml). The dried (Na_2SO_4) extract was concentrated and the residue was eluted from a column (25 × 2 cm) of silicic acid using chloroform to give crude CP in fractions 50-100 (10-ml fractions). Purification was effected by TLC on a 20 × 20 cm plate using chloroform-methanol (19:1), location of the major band with I_2-vapor, and elution of the product from the silicic acid in the appropriate zone with the same solvent mixture (25 ml). Crystallization from water (0.3 ml) gave 9.5 mg of pure product (TLC, melting point and mixed melting point, mass spectrum). Optical rotations (table 5) were obtained using a Perkin-Elmer 141 polarimeter.

RESULTS AND DISCUSSION

We reported earlier on the use of a 4-deuterated analog (CP-4-d_2) to differentiate between two possible pathways (initial 4-hydroxylation and initial 6-hydroxylation) which could lead to the observed end-products of metabolism, ie, acrolein and phosphoramide mustard (8). We also showed that metabolism of CP and its 4-d_2 analog by washed rat-liver microsomes proceeded at similar rates (ie, without any appreciable isotope effect) and that the two compounds had virtually identical activities (table 1) against the ADJ/PC6 mouse plasma cell tumor.

The virtual absence of an isotope effect for 4-hydroxylation of CP-4-d_2 indicates that, contrary to expectations, cleavage of the C(4)-D bond is not the rate-determining step in 4-hydroxylation. There is now evidence that only small isotope effects (~1-1.4) are commonly associated with biologic *N*-demethylation (2,9,10), a reaction formally analogous to the cleavage of the *N*(3)-C(4) linkage in CP in the transformation of CP to 4-hydroxycyclophosphamide (4-hydroxy-CP) to aldophosphamide, in contrast to the much higher values (2-10) which have been observed for *O*-demethylation (2). The precise mechanism of biologic *N*-demethylation and related reactions is not yet clear.

In our continuing investigations into the influence of deuterium substituents on the rates of the metabolic steps shown in the major activation and deactivation pathways of CP (fig 1), we have studied the ring-deuterated analogs, CP-4,6-d_4, CP-4,-5,6-d_6, and CP-5-d_2. The synthetic route to these analogs is shown in figure 3. The 4,6-d_4 analog has previously been used as an internal standard for

TABLE 5.—Optical rotation at various wavelengths for CP recovered from human urine (values in parentheses are those for synthetic CP)

Wavelength (nm)	Observed rotation (°)	Specific optical rotation (°) (c 0.95 in methanol)
589	−0.014 (+ 0.001)	−1.5
578	−0.016 (− 0.001)	−1.7
546	−0.017 (+ 0.001)	−1.8
436	−0.029 (− 0.002)	−3.1
365	−0.044 (− 0.002)	−4.6

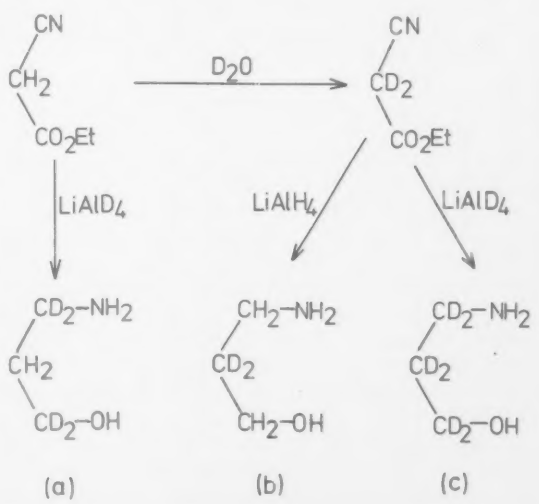


FIGURE 3.—Synthetic routes to deuterated 1,3-propanolamines, precursors of the following CP analogs: (a) 4,6-d_4, (b) 5-d_2, (c) 4,5,6-d_6.

quantitative analysis of CP in human blood and urine by mass spectrometry/stable-isotope dilution (4). In the present study the 4,6-d_4 analog has been used to determine the effect of 4-deuteration upon the rate of formation of 4-keto-CP and carboxyphosphamide (fig 4). The 5-deuterated analogs allow a study of the effect of isotopic substitution on the rate of the β-elimination pathway whereby aldophosphamide is converted into acrolein and phosphoramide mustard (fig 5).

Initially, the relative rates of 4-hydroxylation of CP and its 4-d_2 analog were compared with those of the d_4- and d_6-labeled compounds. A mixture of these four compounds was incubated with washed

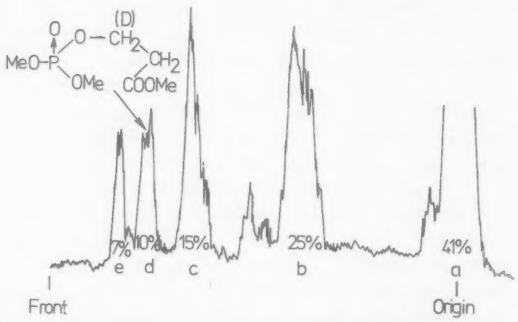


FIGURE 4.—Radiochromatogram scan of a thin-layer chromatogram of CP metabolites produced from a mixture of the drug and its 4,6-d_4 analog after incubation for 30 mins with 9000 g_{av} supernatant. Methylated products developed in ethyl acetate (3 times). The structure for component (*d*) is shown. Component (*a*) was unidentified immobile material. Other identified components were: (*b*) CP, (*c*) a mixture of phosphoramide mustard, *O*-methyl and *O,N*-dimethyl derivatives with carboxyphosphamide methyl ester, and (*e*) 4-keto-CP, *N*(3)-methyl derivative.

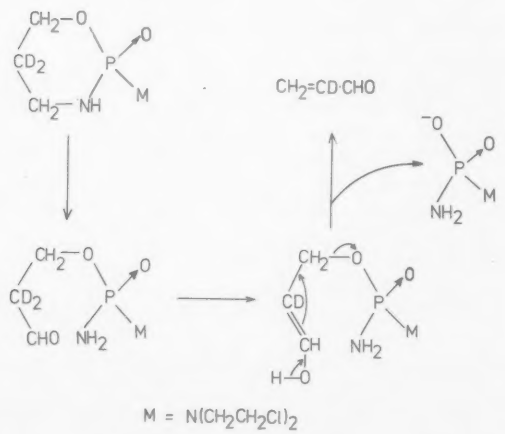


FIGURE 5.—Formation of acrolein-2-d from CP-5-d_2.

microsomes and cofactors under the conditions of the previous experiment (8). The addition of a trace of 5-^{14}C-CP enabled the extent of metabolism of CP to be monitored. Each analog can be distinguished in the mass spectra of mixtures by the different m/e values for structurally equivalent ions (table 2). Since no major fragmentation involves cleavage of a C-D bond, it was found that the relative intensities of the different fragments accurately reflected the molar proportions of each parent compound in mixtures. The ions $[M\text{-}N(CH_2CH_2Cl)_2]^+$ were the most useful in determining the relative abundance of each component since only a small correction (2%) was needed, where appropriate, for the contribution of isotopic (mainly 2-^{13}C-containing) species. The corrected intensity ratios for m/e 120:122:124:126 gave a direct measure of the relative proportions of d_0-, d_2-, d_4-, and d_6-labeled CPs in the initial mixture and in the mixture of CPs isolated after incubation for 20 minutes. From these values the extent of metabolism of each analog could be calculated from the known value for CP, determined from the consumption of initially added radioactively labeled CP (8). The results (table 3) show a small decrease in the extent of metabolism in parallel with increasing deuterium content, but this decrease is not likely to cause any marked difference in biologic activity between CP and the d_4 or d_6 analogs.

Isotope Effects in the Formation of 4-Keto-CP and Carboxyphosphamide

Steps occurring after 4-hydroxylation in the metabolic scheme (fig 1) are also potentially modifiable by ring deuteration. Thus, the 4,6-d_4 analog can be used to study the influence of 4-deuteration upon the conversion of 4-hydroxy-CP and of aldophosphamide into 4-keto-CP and carboxyphosphamide respectively. A mixture of CP (again containing a trace of the 5-^{14}C-labeled drug to facilitate monitoring of products) and the 4,6-d_4 analog was incubated with rat-liver 9000 g_{av} supernatant, which promotes the further oxidation (7) at C-4 to 4-keto-CP, carboxyphosphamide, and the corresponding 6-d_2 analogs. These products were detected by mass spectrometry as their *N*(3)-methyl and methyl-ester derivatives respectively after treatment of the reaction products with ethereal diazomethane and separation by TLC. Repeated development (fig 4) separated *N*-methyl-4-keto-CP from a component having a mass spectrum consistent with the structure (fig 4) previously reported for a methylation product of a urinary metabolite of CP

from sheep (11). Thus, the mass spectrum contained, *inter alia*, the signals at m/e 212 ($M^{+\cdot}$), 181 ($[M\text{-}OCH_3]^+$), and 153 ($[M\text{-}CO_2CH_3]^+$) with the appropriate relative intensities, together with corresponding ions for the dideuterated derivative at m/e 214, 183, and 155. The ratio 1.9:1 between the protium and deuterium forms was similar to that recorded (see below) for carboxyphosphamide.

Carboxyphosphamide methyl ester was not separated from phosphoramide mustard methyl ester but was readily distinguished therefrom in the mass spectrum of the mixture by characteristic signals at m/e 166 and 168 ($[M\text{-}N(CH_2CH_2Cl)_2]^+$) which are the base peaks for carboxyphosphamide methyl ester and its 6-d_2 analog respectively (for the mass spectrum of phosphoramide mustard methyl ester *see* ref 7). The ratio between the ion intensities for the base peaks $[M\text{-}CH_2Cl]^+$ in the mass spectrum of the mixture of the *N*(3)-methyl derivatives of 4-keto-CP (m/e 239) and its 6-d_2 analog (m/e 241) after correction for the isotopic contribution to the latter signal (^{37}Cl-containing ion, protium form), indicates the ratio of d_0:d_2 species to be 2.2:1. For carboxyphosphamide, the corresponding ratio (m/e 166:168) was 1.8:1.

During the period of incubation (30 mins) 75% of the CP was metabolized, and the ratio of CP:CP-4,6-d_4, initially 100:102, became 100:120, a change very similar to that occurring when washed microsomes were used (table 3) and indicative of only a small isotope effect for the initial hydroxylation reaction.

Isotope Effect for the Formation of Acrolein and Phosphoramide Mustard

A key step in the metabolism of CP which might be modified by deuterium substitution at C-5 (fig 5), is the β-elimination step whereby aldophosphamide is converted into acrolein and the highly toxic phosphoramide mustard. Whereas the metabolic transformations previously discussed are undoubtedly enzymatic, the β-elimination reaction is probably a spontaneous chemical transformation at physiologic pH.

The procedure followed for determining the isotope effect for this process was based on that previously used by Alarcon and Meienhofer (12) to trap acrolein released after microsomal or chemical oxidation of CP. A mixture of CP and its 5-d_2 analog was incubated with rat-liver microsomes and cofactors for 30 minutes to obtain optimal conversion into the 4-hydroxy derivatives. The extent of metabolism was 60% and no further metabolism of CP occurred during the subsequent passage of air to remove released acrolein. The initial ratio of CP:CP-5-d_2 (0.98:1.00) was also maintained (within 2%). The ratios between the molecular ions (m/e 236, 237) in the mass spectra of the mixtures of the 2,4-dinitrophenylhydrazones of acrolein and acrolein-2-d were corrected for the contribution to m/e 237 of isotopic (mainly ^{13}C-containing) ions corresponding to the unlabeled derivative (11% of m/e 236). The resulting ratios of acrolein:acrolein-d for the successive times of collection (table 4) show a gradual decrease. This was expected if an isotope effect is operative, since the more rapid decay of aldophosphamide compared with its deuterated analog will enrich the mixture relatively in aldophosphamide-d_2 (or the precursor, 4-hydroxy-CP-5-d_2).

Voelcker et al (13) have determined the half-life for the conversion of 4-hydroxy-CP to aldophosphamide to phosphoramide mustard + acrolein under conditions (pH 7, phosphate buffer, 37°C) very similar to those reported here. From these values it is clear that the deviation from unity of the ratio of aldophosphamide to aldophosphamide-d_2 (fig 5) will be small for the period up to and including the first 10 minutes of acrolein collection, since (*a*) 4-hydroxy-CP will be formed, as well as decomposing, during the initial 30-minute period, and (*b*) the overall conversion of 4-hydroxy-CP to phosphoramide mustard + acrolein is relatively slow (half-life = 107 mins). Thus, the initial ratio of acrolein:acrolein-d (5.3) is close to the true isotope effect k_H/k_D for the decay of the d_2 analog by β-elimination.

Antitumor Test on CP and its Deuterated Analogs

The isotope effects determined for the metabolic transformations of CP are now considered in relation to the results of antitumor tests of CP and its deuterated analogs against the ADJ/PC6 mouse plasma cell tumor (table 1). Although there are chemically significant isotope effects for the detoxification pathways leading to 4-keto-CP and carboxyphosphamide consequent on 4-deuteration, CP and its 4-d_2 analog do not have significantly different antitumor activity (8). The biologic significance of these detoxification pathways in relation to the observed isotope effects has been further discussed elsewhere in this issue (14). The d_4 analogs (CP-4,6-d_4 and a side-chain labeled analog) differed slightly from CP, possibly because of metabolic transformations involving cleavage of the C(6)-H(or -D) or C(1′)-H(or -D) bonds, eg, *N*-dechloroethylation (7, 11). Only the 5-deuterated analogs show marked differences compared with CP. They show a 7-13-fold fall in tumor toxicity (increase in ED90) which

could be accounted for on the basis of the large isotope effect which retards the β-elimination pathway leading to acrolein and phosphoramide mustard, and thus confirms the presumed role of the latter compound as the cytotoxic metabolite of CP mainly responsible for the antitumor effect of the drug in vivo.

Since retardation of the β-elimination adversely affects the antitumor activity of CP derivatives, the effect of accelerating the β-elimination is being investigated. In this context, derivatives such as 5-trifluoromethylcyclophosphamide are being studied.

Quantitative Analysis of CP and 4-Keto-CP in Human Plasma and Urine

As part of a study of the use of deuterium-labeled compounds for the quantitative analysis of CP and its metabolites in human body fluids using mass spectrometry/stable-isotope dilution, we have previously reported on the quantitation of CP in human blood and urine using a direct-insertion technique and CP-4,6-d_4 as internal standard (4). The potential advantages and disadvantages of direct-insertion mass spectrometry compared with the more conventional gas chromatography-mass spectrometry have been discussed elsewhere (4,15). We now report on the quantitation of CP and 4-keto-CP in plasma and the simultaneous determination of these components in urine.

Details for the synthesis of the appropriate d_4-labeled standards are given elsewhere (6) but the route to the analogs described here is summarized in figure 6. Oxidation of CP with $KMnO_4$ (16) as a convenient route to 4-keto-CP-d_4 required a side-chain labeled precursor shown in figure 6. Thus,

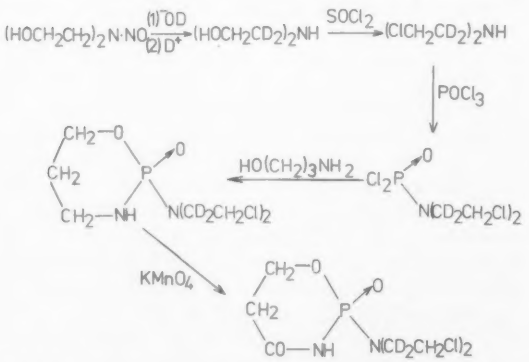


FIGURE 6.—Synthetic route to deuterated internal standards for quantitative analysis of CP and 4-keto-CP.

the ratio (determined by mass spectrometry) between 4-keto-CP and its 5,6-d_4 analog produced by $KMnO_4$ oxidation of a mixture of CP and the ring-labeled d_6 analog was indicative of a large isotope effect (about 8) for the overall conversion of CP-d_6 to 4-keto-CP-d_4, thus disfavoring the use of the ring-labeled precursor in the synthesis of a d_4-labeled 4-keto-CP.

For simultaneous determination of CP and its 4-keto derivative in extracts of plasma or urine, conversion of the 4-keto derivative into the more volatile *N*(3)-methyl derivative was necessary; CP was unaffected by the methylation procedure. Thus urinary levels (4 and 24 hrs) of CP and 4-keto-CP were simultaneously determined from the mass spectrum of the diazomethane-treated methanol eluate following column chromatography of the urine (with added standards) on Amberlite XAD-resin. The respective values were 300 and 21 μg/ml for CP and 30 and 6 μg/ml for 4-keto-CP. The lower plasma levels of 4-keto-CP could not be directly determined in this manner, since background signals in the region containing the relevant ion ($[M-CH_2Cl]^+$ for the *N*(3)-methyl derivative, m/e 239) were one tenth of the intensity of the signal (m/e 243, equivalent to 8.5 μg of the d_4 analog) for the added standard. Purification by TLC of the mixture of *N*(3)-methyl derivative and added standard resulted in a tenfold reduction of this background level and permitted quantitation of the 4-keto derivative (fig 2).[7]

The availability of urine samples from CP-treated patients prompted an investigation into the possibility that the metabolism of the drug is stereoselective since CP possesses a chiral center, ie, at phosphorus. Crystalline CP recovered from the urine of a patient had a negative optical rotation (table 1) showing that metabolism was stereoselective. The extent of this selectivity (the pure R and S forms of CP are not yet available for comparison) is currently under investigation.

[7]Quantitation of 4-keto-CP in human urine, but not in plasma, has also been reported elsewhere (6).

REFERENCES

1. GAFFNEY TE, KNAPP D, WALLE T, ET AL. The importance of stable isotope labelled drugs in clinical pharmacology: with illustrations from studies on the disposition of propranolol and nortriptyline in man. South Med J 67:990-1002, 1974.
2. FOSTER AB, JARMAN M, STEVENS JD, ET AL. Isotope effects in *O*- and *N*-demethylations mediated by rat liver microsomes: an application of direct insertion electron impact mass spectrometry. Chem Biol Interact 9:327-340, 1974.
3. TANABE M, YASUDA D, LEVALLEY S, ET AL. The pharmacologic effect of deuterium substitution on 5-*n*-butyl-5-ethylbarbituric acid. Life Sci 8:1123-1128, 1969.

4. JARMAN M, GILBY ED, FOSTER AB, ET AL. The quantitation of cyclophosphamide in human blood and urine by mass spectrometry-stable isotope dilution. Clin Chim Acta 58:61-69, 1975.
5. CONNORS TA, JONES M, ROSS WCJ, ET AL. New platinum complexes with antitumour activity. Chem Biol Interact 5:415-424, 1972.
6. GRIGGS LJ, and JARMAN M. Synthesis of deuterium-labeled analogs of cyclophosphamide and its metabolites. J Med Chem 18:1102-1106, 1975.
7. CONNORS TA, COX PJ, FARMER PB, ET AL. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
8. CONNORS TA, COX PJ, FARMER PB, ET AL. Observations on the mechanism of hydroxylation of cyclophosphamide by rat liver microsomes: the metabolism of cyclophosphamide-4-d_2. Biomed Mass Spectrom 1:130-136, 1974.
9. THOMPSON JA, and HOLTZMAN JL. Deuterium and tritium isotope effects on the microsomal *N*-demethylation of ethylmorphine. Drug Metab Dispos 2:577-582, 1974.
10. ABDEL-MONEM MM. Isotope effects in enzymatic *N*-demethylation of tertiary amines. J Med Chem 18:427-430, 1975.
11. BAKKE JE, FEIL VJ, and ZAYLSKIE RG. Characterization of the major sheep urinary metabolites of cyclophosphamide, a defleecing chemical. J Agric Food Chem 19:788-790, 1971.
12. ALARCON AR, and MEIENHOFER J. Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide. Nature [New Biol] 233:250-252, 1971.
13. VOELCKER G, DRAEGER U, PETER G, ET AL. Studien zum Spontanzerfall von 4-Hydroxycyclophosphamid und 4-Hydroperoxycyclophosphamid mit Hilfe der Dünnschichtchromatographie. Arzneim Forsch 24:1172-1176, 1974.
14. COX PJ, PHILLIPS BJ, and THOMAS P. Studies on the selective action of cyclophosphamide (NSC-26271): inactivation of the hydroxylated metabolite by tissue-soluble enzymes. Cancer Treat Rep 60:321-326, 1976.
15. GARLAND WA, TRAGER WF, and NELSON SD. Direct (non-chromatographic) quantification of drugs and their metabolites from human plasma utilizing chemical ionization mass spectrometry and stable isotope labeling: quinidine and lidocaine. Biomed Mass Spectrom 1:124-129, 1974.
16. JARMAN M. Formation of 4-ketocyclophosphamide by the oxidation of cyclophosphamide with $KMnO_4$. Experientia 29:812-814, 1973.

Studies on the Disposition of Cyclophosphamide (NSC-26271) in Tumor-Bearing Mice by Means of Gas Chromatography-Chemical Ionization-Mass Fragmentography[1, 2]

C. Pantarotto,* A. Martini, G. Belvedere, M. G. Donelli, and A. Frigerio [3, 4]

SUMMARY

The disposition of cyclophosphamide (CP) in mice bearing Lewis lung tumor has been studied by chemical ionization-mass fragmentography using isophosphamide as the internal standard. The drugs were analyzed as their *N*-trifluoroacetyl derivatives in order to increase sensitivity and to avoid column adsorption and decomposition phenomena. The minimum detectable amount was 500 pg/injection. Linearity was found up to microgram amounts of the substance without any interference of endogenous compounds.

[Cancer Treat Rep 60:493-500, 1976]

Cyclophosphamide (CP) has been previously determined in biologic specimens by means of colorimetric, radioisotopic, and mass-spectrometric methods (1-4). More recently a higher degree of sensitivity and specificity was achieved by a gas-chromatographic assay procedure with electron-capture detection that has been set up in our laboratory (5). However, difficulties arise when the drug is to be determined in particular tissues such as the brain because of the high percentage of interference by endogenous substances which require laborious extraction and purification procedures for elimination.

In the last year, chemical ionization (CI)-mass fragmentography (MF) has been employed in the quantitative determination of drugs and their metabolites (6). The greatest advantage of CI over the classical electron impact-mass spectrometry is a reduced fragmentation pattern accompanied by a prominent "quasimolecular ion" $(M+1)^+$. The possibility of focusing on the $(M+1)^+$ ion has prompted us to apply this technique to MF detection and measurements of the *N*-trifluoroacetyl derivative of CP, thus satisfying the requirements of high specificity and sensitivity.

Isophosphamide (IP), an analog of CP with similar antitumor activity (7,8), also forms a stable *N*-trifluoroacetyl derivative and was therefore chosen as an internal standard for quantitation of CP.

MATERIALS AND METHODS

Standards and Reagents

CP, IP, and methylaminochlorobenzophenone and the reagents trifluoroacetic anhydride (Fluka), sodium hydroxide (NaOH), diethyl ether, and ethyl acetate (Carlo Erba) were used.

Gas Chromatography-CI-MF

A Finnigan model 3100 quadruple mass spectrometer equipped with a gas chromatograph and a computer system (model 6000, programmed for MF) was used. The chromatographic conditions were as follows. The column was a glass tube, 1.5 m long and 4 mm internal diameter, packed with 3% SE-30 on Chromosorb Q, 100-120 mesh, conditioned for 1 hour at 250°C (30 ml/min helium flow), 4 hours at 310°C (no helium flow), and 24 hours at 230°C (35 ml/min helium flow). The operating conditions for

[1]Supported by Consiglio Nazionale delle Ricerche contract 72-00207-04.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

Cyclophosphamide and isophosphamide were generously supplied by Asta Werke, Brackwede, West Germany, and methylaminochlorobenzophenone by Ravizza, Muggiò, Milan, Italy.

[3]Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy.

[4]We are indebted to Mr. M. Broggini and Miss M. Mainini for their expert technical assistance.

**Reprint requests to:* Dr. C. Pantarotto, Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea 62, 20157 Milan, Italy.

the analysis were: column temperature, 200°C; injector port temperature, 250°C; and the flow rate of carrier gas (helium for electron impact and methane for CI analysis), 35 ml/minute. The mass spectrometer was set to the following conditions: molecular separator temperature, 250°C; ion source temperature, 100°C; energy of the ionization beam, 70 electron volts; ionization current, 200 microamperes. CI-MF measurements were performed by focusing the instrument on the ions at m/e 359 (isotopic $M+1)^+$, 357 $(M+1)^+$, 323 (isotopic $M+1)^+$-HCl, and 321 $(M+1)^+$-HCl.

Extraction Procedure From Water, Serum, Urine, and Tissues

Depending on the point of the curve, either 500 ng or 5 μg of IP (internal standard), 0.9 ml of 0.1 N NaOH, and 5 ml of diethyl ether were added to 0.1 ml of water, serum, urine, or to 100 mg of mouse tissue (after homogenization). The appropriate amount of internal standard was determined from preliminary experiments to give a suitable ratio of peak areas with respect to CP. The test tubes were subjected to mechanical shaking for 10 minutes and, after centrifugation for 5 minutes at 4°C, 4.5 ml of the organic phase was transferred to a second test tube. It was then dried under a gentle stream of nitrogen at 35°C in a water bath. The alkaline aqueous layer was extracted again with another 5 ml of diethyl ether and after shaking and successive centrifugation, 5 ml of the organic phase was transferred into the corresponding test tubes and dried. The extraction recoveries of CP and IP from water, serum, urine, and tissues are reported in table 1.

N-Trifluoroacetylation Reaction

The dry residue was redissolved in 150 μl of a mixture of trifluoroacetic anhydride and ethyl acetate (1:2 vol/vol) and the stoppered tubes were heated at 70°C in a sand bath for 20 minutes. The samples were dried under nitrogen stream, 50 μl of ethyl acetate was added, and 2 or 3 μl was injected into the gas-chromatographic column. The reaction kinetics of CP and IP *N*-trifluoroacetyl derivatives formation at room temperature (25°C) and at 70°C, and the stability of the *N*-trifluoroacetyl derivatives in relation to time are reported in figure 1. Those measurements were performed using methylaminochlorobenzophenone as an internal marker added after the reaction.

TABLE 1.—Extraction recoveries of CP and IP from water, serum, urine, and tissue samples

Sample	Extraction recovery (%) of— CP (± SE)	IP (± SE)
Water	77.4 ± 0.8	79.2 ± 1.3
Serum	77.0 ± 1.5	77.8 ± 1.0
Urine	77.1 ± 1.3	80.3 ± 1.8
Lung	76.5 ± 0.9	78.3 ± 1.2
Kidney	78.9 ± 1.7	79.7 ± 0.8
Heart	77.0 ± 1.8	79.8 ± 1.3
Spleen	76.9 ± 1.7	79.5 ± 0.7
Brain	75.8 ± 1.1	78.6 ± 1.7
Tumor	77.7 ± 0.9	79.9 ± 1.4

Biologic Measurements of CP in Serum, Urine, and Tissues

By studying the disappearance curve of the drug in tumor-bearing mice it has been demonstrated that this procedure works for in vivo determinations of CP. The experimental tumor employed was Lewis lung carcinoma implanted im in the right limb of C57BL/6J inbred mice (22 ± 2 g) by injecting 0.1 ml of tumor homogenate diluted 1:10; the animals were injected iv with a single dose of 85 mg/kg of CP prepared immediately before administration by dissolving the compound in distilled water. Groups of three animals were killed and biologic samples (serum and tissues) were collected at 1, 5, 10, 15, 20, 40, and 60 minutes after drug injection. The method of residuals was applied for calculating kinetic parameters (9). Urine samples were collected from tumor-bearing mice housed in glass metabolic cages and collections were made 1, 4, and 24 hours after CP administration.

RESULTS AND DISCUSSION

The gas chromatogram of CP and IP as their *N*-trifluoroacetyl derivatives is shown in figure 2. The *N*-trifluoroacetylation reaction was chosen because of the suitable retention time of the derivatives formed and also in order to avoid adsorption on the stationary phase of the gas-chromatographic column and decomposition phenomena. In gas chromatography, underivatized CP undergoes an intramolecular alkylation catalyzed by the slight acidity of the phase. This decomposition is common to underivatized IP (although in lower percentage) therefore comfirming the structure previously proposed by Friedman et al (10) in the aqueous hydrolysis of CP (fig 3).

The identities of these gas-chromatographic peaks were checked by means of gas chromatography-mass spectrometry and the structures were also elucidated by comparing the mass spectra obtained by using electron impact and CI ion sources.

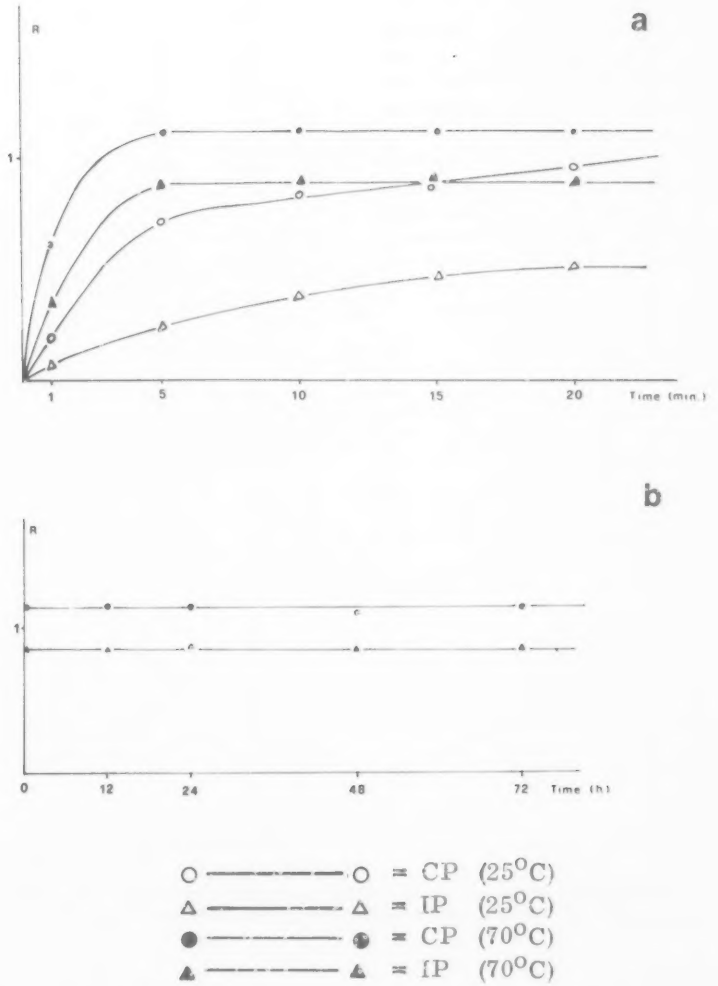


FIGURE 1.—(*a*) Reaction kinetics of CP and IP at room temperature and at 70°C. (*b*) stability of the *N*-trifluoroacetyl derivatives: ordinate = the peak area ratio (R) of CP or IP to the internal marker (methylaminochlorobenzophenone); abscissa = time. These data were obtained from a concentration of 100 ng of CP and IP; quantitation was performed using 0.5 μg of internal marker added after the reaction.

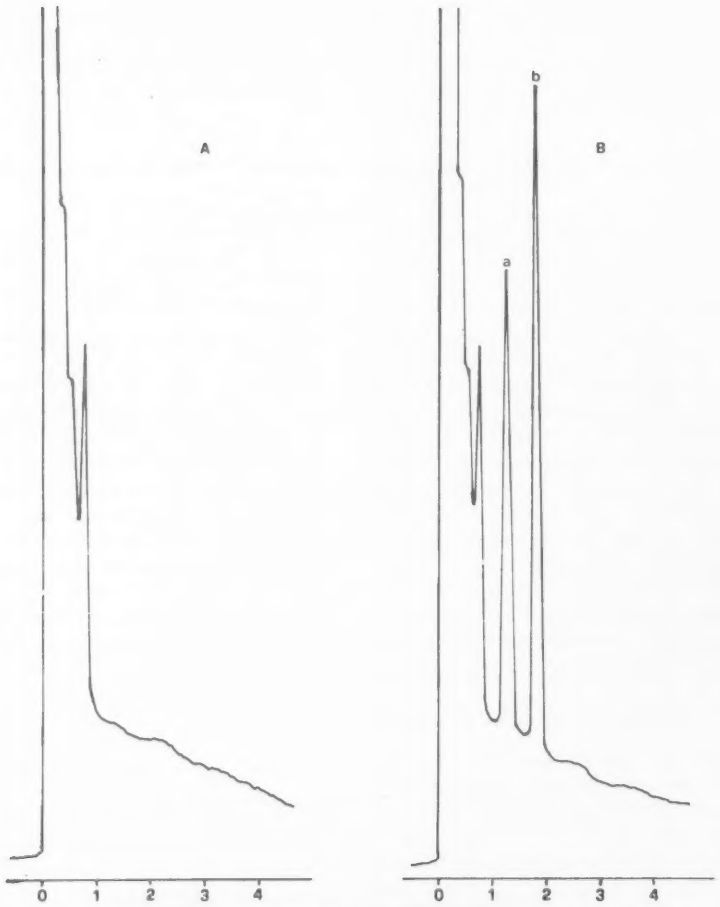


FIGURE 2.—Gas chromatograms of *N*-trifluoroacetylated water extracts: A = blank; B = IP *N*-trifluoroacetate (peak a) and CP *N*-trifluoroacetate (peak b).

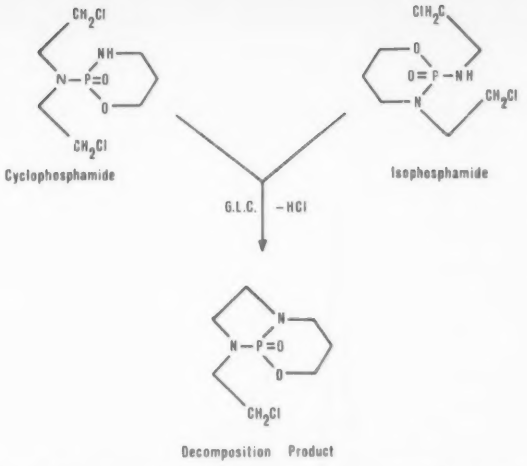


FIGURE 3.—On-column intramolecular alkylation of underivatized CP and IP.

The CI mass spectra of the *N*-trifluoroacetyl derivatives of these drugs are shown in figure 4. According to the principles of the MF technique, in order to attain higher sensitivity it is generally advisable to focus on ions with a high abundance (preferably the base peak and the molecular ion). In the case of CP and IP the fragmentation by electron impact presents molecular ions of a low intensity (~1%), not useful for quantitative determinations; other fragments of lower mass may also be derived from many endogenous substrates and therefore electron impact-MF detection and quantitation will not be able to achieve the level of specificity required to apply this technique to pharmacologic problems. By contrast, in CI-mass spectrometry the base peaks of the *N*-trifluoroacetyl derivatives of CP and IP correspond to the "quasimolecular ions" which are highly specific and satisfy the requirements of MF detection.

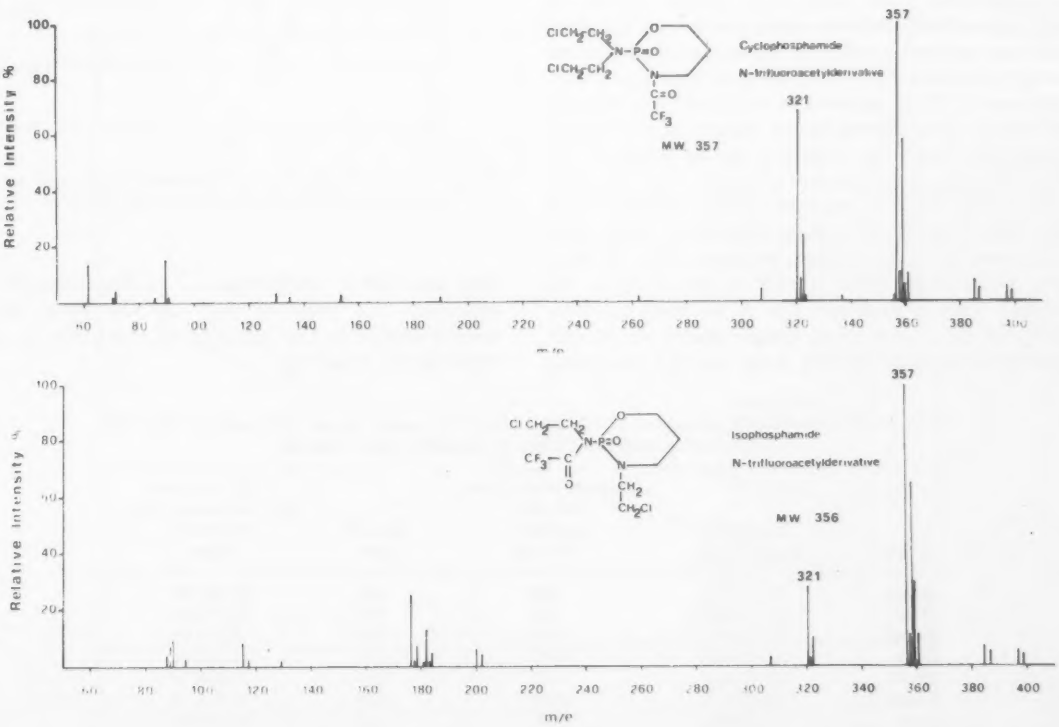


FIGURE 4.—CI mass spectra (reacting gas methane) of CP and IP as their *N*-trifluoroacetyl derivatives.

Figure 5 shows the MF separation of CP and IP extracted from mice brain tissue according to the general extraction procedure described above. Multiple ion detection was performed by focusing the mass spectrometer on the ions at m/e 359 (isotopic $M+1)^+$, 357 $(M+1)^+$, 323 (isotopic $M+1)^+$-HCl, and 321 $(M+1)^+$-HCl, which are common to both substances, and no interference from endogenous substrates was observed.

The calibration curves for CP in serum samples are illustrated in figure 6; the linearity of the method ranges from concentrations of 150 ng/ml to 100 μg/ml. Calibration curves from urine and tissue samples are not reported because they were identical to those of serum due to the same recovery of extraction (*see* table 1). All of these results are valid when IP is determined by utilizing CP as the internal standard.

The possibility of applying this technique to the quantitative evaluation of these alkylating agents in biologic specimens was investigated by measuring the disappearance curves of CP in serum and tissue samples of tumor-bearing mice. The results of the distribution in serum and tissue samples are shown in figures 7 and 8 and the corresponding pharmacokinetic parameters are reported in table 2. Serum and liver levels were found to be in good agreement with the data previously obtained from our group by utilizing a gas-chromatographic assay procedure (5). It can be seen that the concentrations of CP in serum are relatively low in comparison to those found in the organs so that rapid penetration into the tissues must be assumed. CP easily crosses the blood-brain barrier and the peak level in the brain is reached rapidly within 5 minutes; the rate of CP disappearance is lower than that seen in other tissues because after 10 minutes, the concentration of CP in the brain is the highest. This is probably due to the high liposolubility of CP. Lewis lung tumor shows no special ability to concentrate the drug but the relatively long half-life of unchanged CP in this tissue is of interest; this may be due to the poor blood supply and/or to the inability of the tumor to metabolize the drug (11).

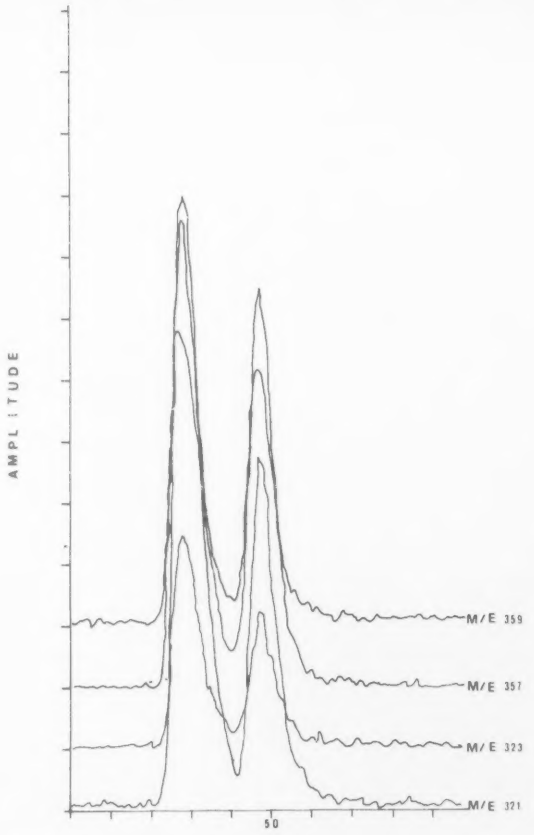


FIGURE 5.—Mass fragmentogram of a brain extract: peak a = IP *N*-trifluoroacetate; peak b = CP *N*-trifluoroacetate.

TABLE 2.—Pharmacokinetic parameters of CP (85 mg/kg iv) in serum, tissues, and tumor of C57BL/6J mice bearing Lewis lung tumor 25 days after tumor injection

Sample	Peak value (μg/ml or g)	Concentration × time (0-60 min) (μg/min/ml or g)	Half-life (min)	Upper and lower 95% confidence limits of half-life (min)
Serum	72 (1 min)	549	9.24	11.63-7.66
Liver	110 (1 min)	873	7.70	10.77-5.98
Kidney	138 (1 min)	1184	7.00	9.13-5.67
Lung	214 (1 min)	1671	8.15	11.68-6.26
Heart	135 (1 min)	1155	9.90	12.63-8.13
Spleen	122 (5 min)	1187	8.66	13.27-6.43
Brain	86 (5 min)	1651	10.66	11.49-9.94
Tumor	21 (5 min)	507	15.75	16.59-14.98

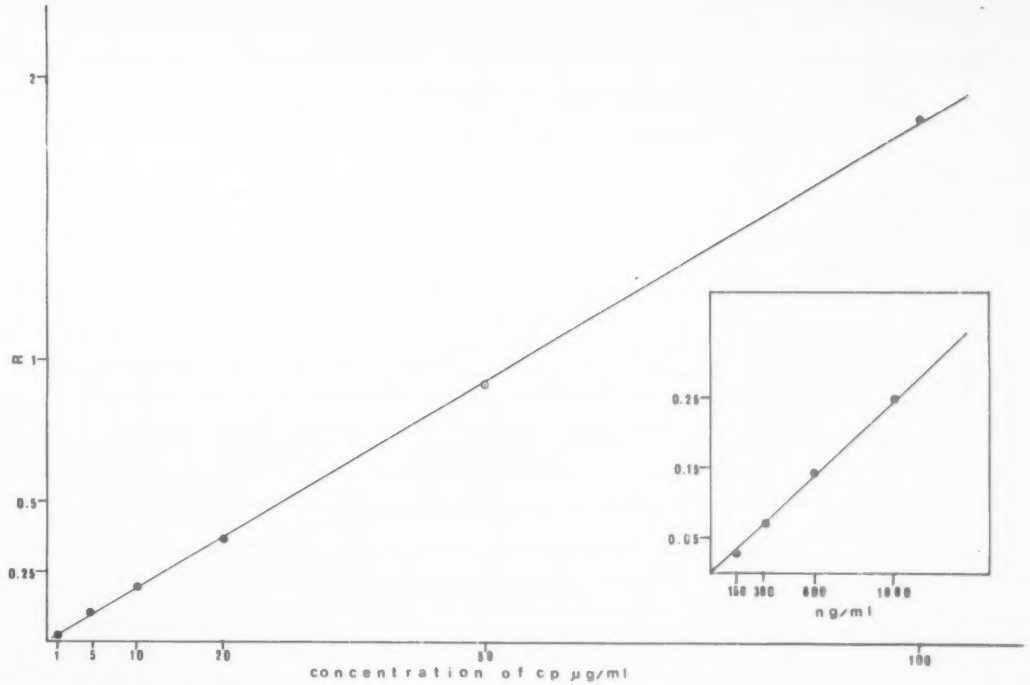


FIGURE 6.—Standard calibration curve of CP with the internal standard IP; R = peak area ratio of CP to IP.

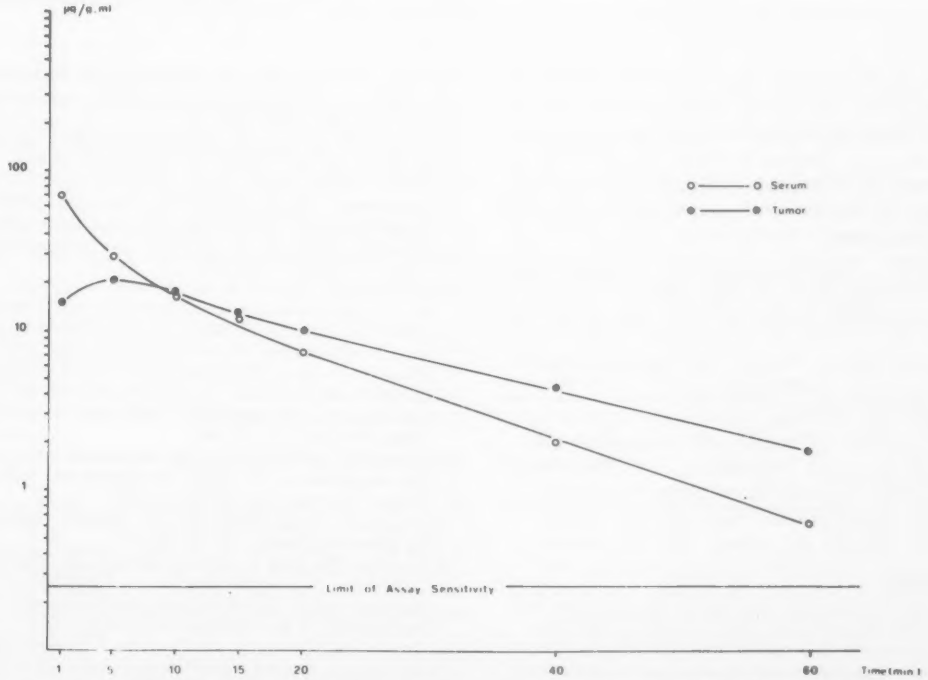


FIGURE 7.—CP levels in serum and in Lewis lung tumor at different times after iv injection of 85 mg/kg; each point is the average of 3 determinations.

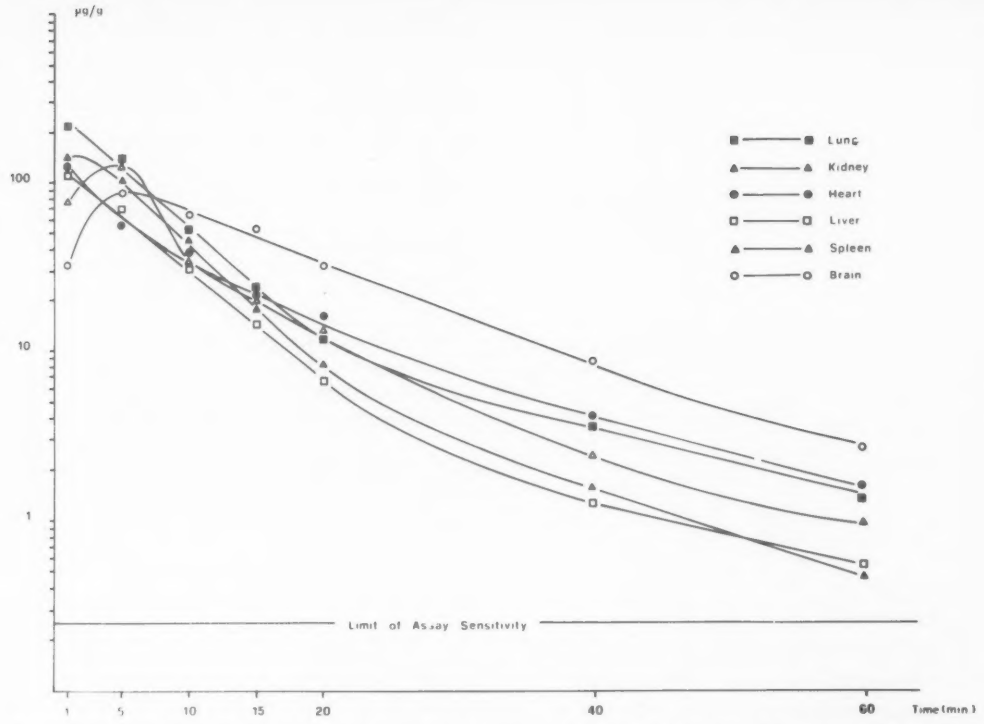


FIGURE 8.—Tissue concentrations of CP at different times after iv injection of 85 mg/kg. Each point is the average of 3 determinations.

The excretion of CP in the urine of C57BL/6J mice bearing im Lewis lung tumor can be seen in table 3. Both the excretion rate and the cumulative excretion are quite similar to those observed by us in normal C57BL/6J mice (5). It may be noted that the presence of the tumor does not seem to modify the kidney function.

TABLE 3.—Urinary excretion of CP (85 mg/kg iv) in C57BL/6J mice bearing im Lewis lung tumor

Time range (hr)	μg ± SE	% administered dose ± SE
0-1	119 ± 9	4.98 ± 0.36
1-4	17 ± 2	0.72 ± 0.09
4-24	1 ± 0.5	0.05 ± 0.02
Cumulative	137	5.6

REFERENCES

1. FRIEDMAN OM, and BOGER E. Colorimetric estimation of nitrogen mustard in aqueous media. Anal Chem 33:906-910, 1961.
2. HIRATA M, KAGAWA H, and BABA M. Cyclophosphamide. I. Determination and degradation kinetics in aqueous media. Annu Rep Shionogi Res Lab 17:107-113, 1967.
3. GRAUL EH, SCHAUMLÖFFEL E, HUNDESHAGEN H, ET AL. Metabolism of radioactive cyclophosphamide. Animal tests and clinical studies. Cancer 20:896-899, 1967.
4. DUNCAN JH, COLVIN OM, and FENSELAU C. Mass spectrometric study of the distribution of cyclophosphamide in humans. Toxicol Appl Pharmacol 24:317-323, 1973.
5. PANTAROTTO C, BOSSI A, BELVEDERE G, ET AL. Quantitative GLC determination of cyclophosphamide and isophosphamide in biological specimens by means of gas chromatography. J Pharm Sci 63:1554-1558, 1974.
6. PANTAROTTO C, MARTINI A, BELVEDERE G, ET AL. Application of gas chromatography-chemical ionization mass fragmentography in the evaluation of bases and nucleoside analogs used in cancer chemotherapy. J Chromatogr 99:519-527, 1974.
7. SHEEF W. Problems, experience and results of clinical investigations with ifosfamide. *In* Advances in Antimicrobial and Antineoplastic Chemotherapy. Baltimore, University Park Press, 1972, vol 2, pp 797-800.
8. ALLEN LM, and CREAVEN PJ. Gas chromatographic method for the determination of plasma isophosphamide (NSC-109724). Cancer Chemother Rep 56:721-723, 1972.
9. WAGNER JG. Biopharmaceutics and Relevant Pharmacokinetics. Hamilton, Hamilton Press, 1971.
10. FRIEDMAN OM, BIEN S, and CHAKRABARTI JK. Studies on the hydrolysis of cyclophosphamide. I. Identification of *N*-(2-hydroxyethyl)-*N'*-(3-hydroxypropyl)ethylendiamine as the main product. J Am Chem Soc 87:4978-4979, 1965.
11. DONELLI MG, COLOMBO S, and GARATTINI S. Experiments aiming at demonstrating microsomal drug metabolism in the tumor tissue. Eur J Cancer 8:181-183, 1972.

Qualitative and Quantitative Investigations of Cyclophosphamide (NSC-26271), Cyclophosphamide Metabolites, and Related Compounds by Field-Desorption Mass Spectrometry[1,2]

H.-R. Schulten[3,4,*]

SUMMARY

The potential of field-desorption mass spectrometry (FD-MS) coupled with photoplate detection for qualitative and quantitative studies on cyclophosphamide (CP) and its metabolites is reviewed. The characteristic features of this ionization mode are minimal fragmentation of the molecular ion coupled with the capacity to produce such ions from thermally unstable or nonvolatile samples. Since CP is itself extensively fragmented in the electron-impact ionization mode, and since it possesses both thermally unstable (eg, 4-hydroxycyclophosphamide, carboxyphosphamide) as well as ionic and hence nonvolatile metabolites (eg, phosphorodiamidic acid), the FD technique has potential value for the detection and quantitation of the drug and its metabolites in underivatized mixtures. Some of the factors which influence the reproducibility of FD spectra are enumerated. In order to improve the FD technique for quantitative results a device for emission-controlled FD was constructed and its use is described. Three different modes of operation are discussed using the FD spectra of CP conjugates typical for each. Preliminary investigations into the use of the d_6 analog of CP for quantitation of the drug by FD-MS are also reported.

[Cancer Treat Rep 60:501-507, 1976]

Molecular-weight (MW) determinations of thermally labile and nonvolatile substances by field-desorption mass spectrometry (FD-MS) have established the method as a standard tool in mass spectrometric analysis (1). The main reasons for the analytic capacity of FD-MS in this respect are:

a. In the ionization process of a molecule M in the high field (1-5 × 10^7 V/cm) only a small amount of electronic excitation energy is transferred (a few tenths of an eV). Thus, consecutive fragmentation of the molecular ion $[M]^+$ is strongly reduced.

b. Since the samples need not be vaporized for analysis, a crucial requirement for all conventional mass spectrometric techniques, ionic desorption of the intact molecular ion can be performed under relatively small thermal stress.

c. Due to the high ionization efficiency of FD-MS the sensitivity of the method lies, for low resolution instruments ($M/\Delta M \simeq 350$), between 10^{-13} and 10^{-10} coulomb/μg. This requires, however, very careful sample handling and loading onto the surface of the FD emitter wires.

d. In fulfillment of the need for high sensitivity in biomedical and environmental research, the low sample amount for measurement of the $[M]^+$ and/or $[M+H]^+$ peaks by FD-MS lies between 10^{-6} and 10^{-11} g.

e. Moreover, since the sample is applied to the emitter from a solution it is essential that low concentrations (~10^{-8} M) give useful FD spectra (ie, unambiguous molecular ions) when a modified syringe technique is employed.

[1]Supported by the Deutsche Forschungsgemeinschaft, the Landesamt für Forschung des Landes Nordrhein-Westfalen, and the Fonds der Deutschen Chemischen Industrie.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Institute of Physical Chemistry, University of Bonn, West Germany.

[4]I wish to thank Professors N. Brock and H.-J. Hohorst for the generous supply of the synthetic compounds. The collaboration of Professor H. Ringsdorf and Dr. M. Przybylski on the field-desorption investigations of the cyclophosphamide conjugates is gratefully acknowledged. I am very grateful to Dr. M. Jarman for his help in preparing this manuscript and for the supply of the d_6-cyclophosphamide.

*Reprint requests to: Dr. H.-R. Schulten, Institute of Physical Chemistry, University of Bonn, 53 Bonn, Wegelerstrasse 12, West Germany.

f. In cases where the available sample is in the microgram range, high-resolution FD-MS can be performed (2,3). A knowledge of the elemental composition of the molecular ion and its fragments can considerably facilitate the identification of a chemical species.

g. Photographic detection of the produced FD ions has been preferred for high-resolution work. The integrating function of the recording device (photoplates) for all ions that are generated during the whole desorption time allows a fractionated desorption to be made. If a single homogenous compound is investigated, the ions which desorb in selected time/temperature intervals can be recorded on separate tracks of the photoplate. Simultaneously, the emitter heating current (ehc) is plotted versus the desorption time (thermogram). This additional information about the desorption kinetics in the high field can be used for identification of a chemical species as well as for the fundamental understanding of the desorption process. If, alternatively, a mixture is studied, partial fractionation of components of slightly differing volatilities in the mixture can be carried out giving mainly molecular ions and strongly reduced fragmentation. Thus, highly selective and informative spectra are obtained.

h. Because of this unique capacity for analytic resolution of mixtures and gentle ionization of polar substances, FD-MS enables direct MW determinations on samples from biologic sources without derivatization. The ability of the method to analyze pre-purified extracts (eg, from biomedical investigations) will reduce the need for elaborate separation techniques which involve time and loss of compound.

These characteristic features of the relatively new method led to a widespread use in the analysis of model and unknown compounds.

The focal points in the analytic application of FD-MS are: (*a*) metabolic and nonmetabolic products of drugs (4) and environmental chemicals (5,6); (*b*) high MW organic and inorganic salts (7); (*c*) complex mixtures of natural products, drugs, and pesticides; and (*d*) macromolecules (MW >20,000) which have been investigated by controlled thermal degradation in the high field by pyrolysis-FD-MS (8).

RESULTS AND DISCUSSION

Qualitative Analysis by FD-MS

Concurrent with the improvements of the FD technique a major aim in our research program has been the utilization of FD-MS for analytic problems in cancer research. *N,N*-bis(2-chloroethyl)-aniline and *N,N*-bis(2-hydroxyethyl)aniline, a structurally simple antitumor alkylating agent and its hydrolysis product, were chosen as the first model substances to test the suitability of FD-MS as a technique for investigating the mode of action of this class of cytostatic agents in conjunction with other analytic methods (9). Also, the electron impact (EI) and FD mass spectra of substituted five- or six-membered ring sultams, some of which have been reported to possess antitumor effects against Ehrlich ascites tumors, were compared (10). The main fragmentation pathways for sultams carrying polar substituents such as dicarboxylic acids and their monoamides and diamides were established in both ionization modes and interpreted. Further purine antagonists, such as 1-methyl-4-nitro-5-(*S*-glutathionyl) imidazole (azathioprine) and some of its metabolites, were investigated using FD-MS. The FD spectra of glycine, cysteine, and glutathione conjugates of this widely used immunosuppressive drug were superior to those obtained by EI-MS because they showed intense molecular ions ($[M+H]^+$ ions) as well as characteristic fragments (11). In addition, the mass spectral behavior of mutagenic and cocarcinogenic substances has been explored (12). In general, however, FD studies in cancer research were concentrated on alkylating antitumor agents such as cyclophosphamide (CP) (I), isophosphamide, trophosphamide, and some metabolites of CP. Recently, the FD spectra of 4-ketocyclophosphamide (4-keto-CP) (II), carboxyphosphamide (III), and *N,N*-bis(2-chloroethyl)phosphorodiamidic acid (IV) (as the cyclohexylammonium salt) have been published (13). From these results it became clear that FD-MS is particularly well suited for the analysis of the polar underivatized compounds III and IV. In all cases high molecular ion intensities were obtained and in addition, by thermally/field-induced fragmentation, diagnostic fragment ions could be produced.

The mechanisms of activation and detoxification of CP have been extensively investigated. It appears that one important intermediate in the in vivo activation pathway is 4-hydroxycyclophosphamide (4-hydroxy-CP). We have therefore studied the high-resolution FD spectra of the synthetic compound (fig 1). The molecular ions at m/e 276.020 ($C_7H_{15}O_3PCl_2$) are the base peak of the FD spectrum. Since an aprotic solvent was used (CCl_4), protonation from the solvent can be excluded. However, the relatively high abundance of the $[M+H]^+$ ion (m/e 277), ~21%, indicates that intermolecular protonation takes place. This is confirmed by the occurrence of an $[M-H]^+$ ion (m/e 275)

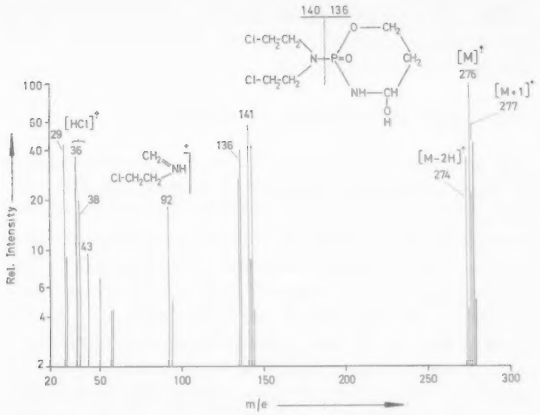


FIGURE 1.—FD mass spectrum of 4-hydroxy-CP. Solvent: carbon tetrachloride, emission-controlled FD-MS, threshold 5 × 10^{-8} A, BAT 11 mA ehc, exposure time 10 mins, temperature range corresponding to 0-30 mA ehc.

and a strongly pronounced $[M-2H]^+$ ion at m/e 274 which are probably produced in a high-field process. From the FD spectrum alone a decision cannot be made as to whether small impurities of 4-keto-CP (MW 274) were present in the synthetic samples or whether oxidation occurred during sample handling and transfer to the emitter. Because of our limited knowledge of the field processes of large organic molecules and possible chemical changes of reactive compounds during emitter coating and transfer into the ion source, the interpretation of FD spectra might presently be difficult in some cases. Therefore, investigations of model compounds are not only justified but necessary (1). But, in addition, studies of the basic principles of the processes in high-field chemistry using relatively simple test substances appear to be a prerequisite for reliable interpretation of the FD data obtained (14).

Other ions in the FD spectrum of the 4-hydroxy-CP derivative (fig 1) are readily assigned. Thus, m/e 136 and 141 are generated by direct bond cleavages and protonation. This fission of a molecular ion into two complementary charged particles is a frequently observed phenomenon in FD-MS. P-N cleavage to give the *N*-mustard and oxazaphosphorine moieties is a feature of the FD spectra of other CP metabolites and analogs and has greatly simplified the interpretation of these spectra. In addition, some ions of minor significance such as $[HCl]^+$ and $[C_3H_7NCl]^+$ are generally recorded in the lower mass range.

An analytically important feature of FD-MS is its use in investigations of salts (7,15). The mass spectrometric analysis of this class of compounds by other conventional methods is more difficult or sometimes impossible due to the requirement of volatilization prior to ionization. In figure 2 the FD spectrum of *nor*-nitrogen mustard is shown. In contrast to the general experience in FD-MS, the molecular ion (fig 2, M = C + A, where C is the cation and A is the anion) of this compound is absent. The energetically and kinetically favored process of cluster formation, however, generates ions from which the molecular structure can be easily derived. The base peak of the spectrum is the *N*-mustard ion at m/e 141.011 ($C_4H_9NCl_2$). The cation at m/e 142 and its fragments due to the loss of hydrochloric acid (m/e 106) and CH_3Cl (m/e 92) are also of relatively high intensity. In the low mass range, intense ions for hydrochloric acid at m/e 35.977 ($H^{35}Cl$, 72% relative intensity) and at m/e 37.974 ($H^{37}Cl$, 30% relative intensity) are recorded.

Likewise, in the FD spectrum of the mixture of CP and some of its metabolites (fig 3), the cyclohexylammonium salt of phosphorodiamidic acid (IV) showed high relative abundances for the cation and clearly detectable $[M+C]^+$ cluster ions (13). It is of interest to determine whether by FD-MS it is possible to conveniently run mixtures of parent drugs and drug metabolites, especially when the components are of widely differing polarities. To this end an equimolar mixture of CP, II, III, and IV was prepared, and the FD mass spectrum was run. One exposure of the photoplate was made for the range of ehcs from 0 to 30 mA. The spectrum in figure 3 was qualitatively the sum of each individual FD spectrum of these compounds.

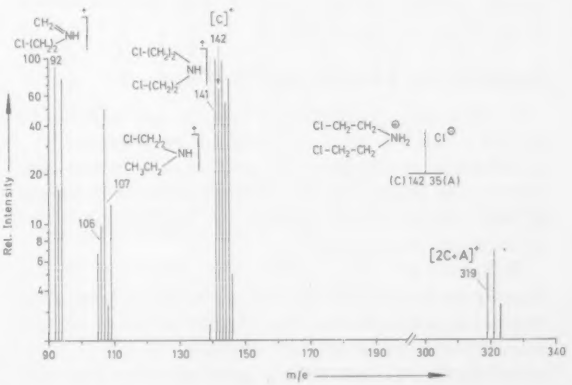


FIGURE 2.—FD mass spectrum of *nor*-nitrogen mustard. Solvent: methanol, emission-controlled FD-MS, threshold 5 × 10^{-8} A, BAT 8 mA ehc, exposure time 9 mins, temperature range corresponding to 0-30 mA ehc.

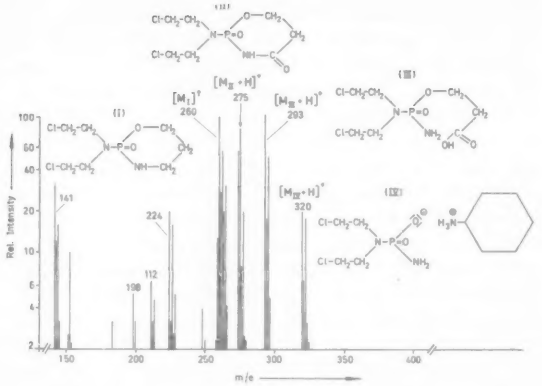


FIGURE 3.—FD mass spectrum of a mixture of I, II, III, and IV. A solution/suspension of 50 μg of each compound in 10 μl acetone was used in this experiment. The amount of sample adsorbed on the emitter surface and consumed in one high-resolution (~15,000) FD mass spectrum was estimated and calculated to be $< 10^{-7}$ g.

Although desorption occurs from the solid adsorbed state, interaction between the different compounds of the mixture is amazingly small. The molecular ions $[M_I]^+$ or protonated molecules $[M_{II,III,IV}+H]^+$ are the most prominent ions in the spectrum. Although the FD spectrum in figure 3 reflects qualitatively the composition of the synthetic mixture, there are clearly marked differences in the ionization efficiencies in FD for the components of this equimolar mixture. Hence, the use of FD-MS for quantitative biomedical and pharmacokinetic studies depends on the development of a reliable calibration method. This involves careful studies of the parameters which influence the reproducibility of FD spectra as well as improvements of the instrumentation for the recording techniques of these spectra.

Quantitative Analysis by FD-MS

In principle, quantitative results are difficult to obtain using FD-MS for the following reasons:

Influence of the field-ion emitter.—Accurate control of the shape, surface, distribution, and length of the micro-needles (grown by high-temperature activation) is not possible.

Influence of the sample.—The behavior of the sample is controlled by the quantity applied, the mode of application, the characteristics of the substance, and the solvent used and its pH.

Influence of desorption parameters.—The FD spectra obtained are governed also by the temperature of the sample and the emitter during desorption, the applied field strength, and the desorption rate achieved.

These three groups of factors are interrelated and a basic understanding of their relative roles has not yet been attained.

Temperature dependence of FD spectra

In general, an increase in the ehc (ie, an increase in sample temperature) is accompanied by tendency towards increased thermal and field-induced fragmentation. The theoretical aspects of this process have been discussed elsewhere (1). The present report is concerned with the practical consequences of this effect as exemplified in the FD spectra (fig 4) of an aldophosphamide derivative (aldophosphamide propanediol-1,3-acetal) (16). The spectrum (*b*) taken at the higher ehc is qualitatively similar to that (*a*) obtained at lower sample temperature, but shows additional peaks (eg, m/e 151, 185, 212, 238, 253, 266), presumably due to pyrolytic processes. Also, the common peaks show, in some cases, drastic changes in intensity after going to a higher sample temperature.

Emission-controlled FD-MS

Besides the absolute temperature of the sample, the rate of desorption from the emitter surface has a strong influence on the appearance of the spectrum. A device has therefore been designed (fig 5) which allows an automatically controlled desorption. Reproducible time/temperature desorption profiles are obtained by setting a threshold measured at the cathode for the total emission of the field-ion emitter. For the present experiments the

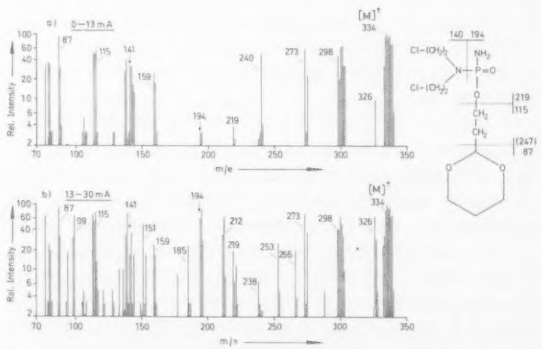


FIGURE 4.—FD mass spectrum of aldophosphamide propanediol-1,3-acetal. Solvent: methylene chloride, BAT 10 mA ehc, emission-controlled FD-MS (a) threshold 2×10^{-8} A, exposure time 15 mins, temperature range corresponding to 0-13 mA ehc, or (*b*) threshold 6×10^{-8} A, exposure time 15 mins, temperature range 13-30 mA ehc. The sample consumption for both spectra was 1 μg.

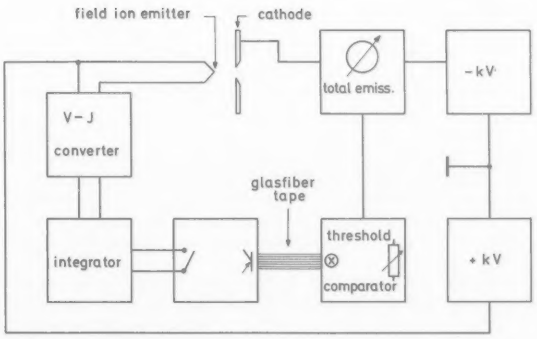


FIGURE 5.—Schematic diagram of a device for emission-controlled FD-MS. Measurements of the total emission (and thresholds) can be performed between 1×10^{-10} and 1×10^{-6} A. The background emission from field-ionized residual gas in the ion source is in general $< 1 \times 10^{-9}$ A.

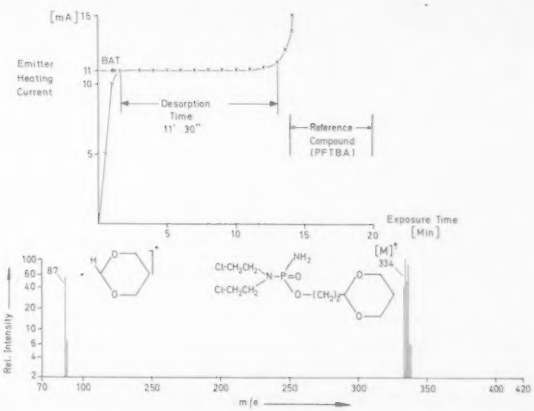


FIGURE 6.—Thermogram of aldophosphamide propanediol-1,3-acetal and FD mass spectrum at a threshold emission of 1.2×10^{-9} A, BAT 11 mA ehc, total exposure time 20 mins. The actual desorption time of the sample was 11 mins 30 secs; 30 secs were consumed for the emitter current regulation in order to reach 11 mA ehc and the rest of the total exposure time was used for the FI (supply from the gasphase) mass spectrum of the reference compound perfluorotributylamine which is needed for mass calibration.

threshold was between 1×10^{-9} and 5×10^{-8} A. The circuit shown in figure 5 allows three modes of operation.

For minimal fragmentation, the threshold current is set at the lowest value ($\sim 1 \times 10^{-9}$ A) producing a detectable ion current (fig 6). In contrast to figure 4, the spectrum of the aldophosphamide propanediol-1,3-acetal shown in figure 6, which was obtained with the described device, shows only one significant fragment. In figure 6 the relationship between the ehc and the desorption time is also shown. From this thermogram, the characteristic best anode temperature (BAT) for the compound is easily derived and this value is dependent on the polarity of the sample. Further, from the actual desorption time derived from the plateau in the thermogram the approximate weight of sample desorbed can be derived, if appropriate calibration curves for that sample, relating sample amount of desorption, are available.

When the threshold current was set at values $> 1 \times 10^{-9}$ A more fragmentation occurred but the spectra are still reproducible for a given threshold current. The intensities of each line on the photoplate for ten determinations of the FD spectrum of aldophosphamide ethanediol-1,2-acetal (16) (fig 7) were reproducible to within 10% (except for very weak lines [< 4% relative intensity] and for lines with saturated blackening).

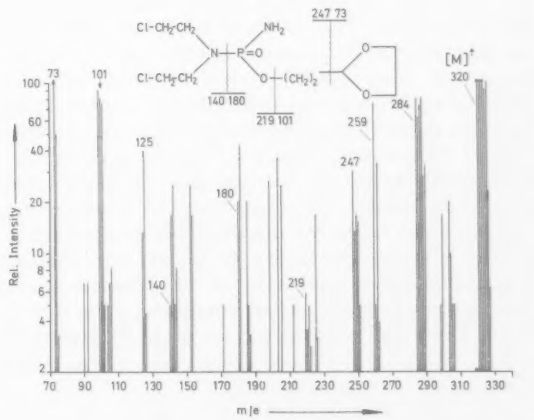


FIGURE 7.—FD mass spectrum of aldophosphamide ethanediol-1,2-acetal. Solvent: 1,2-dichlorobenzene, BAT 11 mA ehc, threshold 1.5×10^{-8} A, exposure time 12-13 mins, temperature range corresponding to 0-30 mA ehc.

A third mode of operation which was most suitable for routine quantitative work was linear programming of the ehc with time. Here, the FD mass spectra of aldophosphamide ethanediol-1,2-acetal showed the molecular-ion group (m/e 320-325) and only a few fragments of minor intensities at m/e 73, 101, and 125. Repeated measurements of 1-μg samples with different but standard FD emitters

(13) gave deviations of only ± 5% relative intensity. However, the procedure of linear programming can only be used when sufficient sample material is available and the physicochemical properties of the molecule are favorable for FD.

Calibration studies with a d_6 analog of CP

The choice of an internal standard for quantitation of CP by FD-MS stable-isotope dilution was governed by the existence of both $[M+H]^+$ ions and ions containing two ^{37}Cl atoms. Thus, the molecular-ion cluster (fig 8) for CP contained ions of m/e 259-265, but there was no detectable ion at m/e 266 (detection limit 2% of base peak at 15,000 resolution). A suitable internal standard is, therefore, a d_6 analog, CP-4,5,6-d_6 (17).

Figure 9 shows the molecular-ion cluster for mixtures of the CP and d_6 analog containing the d_0 and d_6 forms in the molar ratio of 1:0.85 (1:1 with respect to protium and deuterium forms, but the latter contains d_5 and d_6 analogs in the ratio of 15:85). In figure 9a, 1 μg of each component is present on the emitter and recorded by FD-MS with photographic detection. The resulting densitogram was overexposed with relation to the more abundant isotopic peaks. The results with 100 ng of each component are shown in figure 9b. The ratio m/e 260:266 (two ^{35}Cl-containing $[M]^+$ ions for d_0 and d_6-analog) was 100:86 (theory, 100:85) and the $^{35}Cl^{37}Cl$-containing ions (262,268) are approximately in the same ratio. Greater departures from theoretical values are observed for the less intense ions, particularly m/e 264 and 265. At the present resolution (15,000), 100 ng therefore represents a near-optimal value for quantitative determination of CP, but the sensitivity would be enhanced by the use of lower resolution or by different detection systems. In the latter connection, the use of an FD source in combination with a quadruple

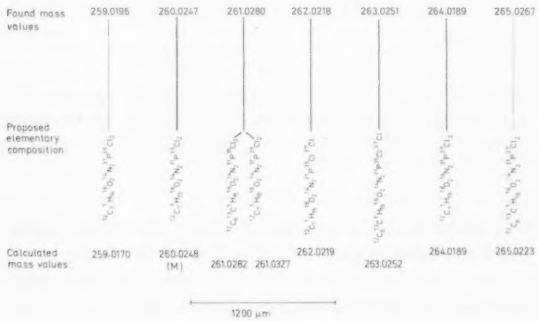


FIGURE 8.—High-resolution FD-MS of the molecular-ion group of CP with photographic detection. Lengths, widths, and distances of the lines are drawn as they appear in a homebuilt densitometer.

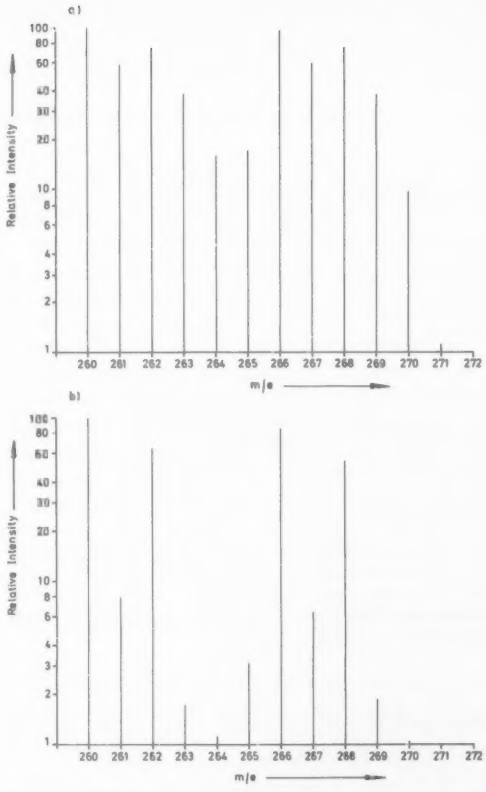


FIGURE 9.—FD mass spectra of a mixture of CP and CP-d_6 (1.00:0.85): (*a*) 1 μg CP; (*b*) 0.1 μg CP. Solvent: acetone, threshold 1×10^{-8} A, exposure time 30 mins.

mass spectrometer and a time-averaging computer is currently under investigation.

EXPERIMENTAL PROCEDURES

All FD spectra were recorded on a modified, double-focusing CEC21-110B mass spectrometer with vacuum-evaporated AgBr plates. The FD ions were generated by high-temperature activated 10-μm tungsten wire emitters. Activation was performed in a tenfold activation cell, the length of the microneedles averaged about 30 μm, and their morphology and distribution were similar to those shown in reference 9. The ionization efficiency of the emitters was checked in the field ionization (FI) mode prior to the FD experiments. In general, 1 μg of the substances was applied from a 10^{-3} M solution with the modified syringe technique (1). In all cases desorption was performed by automatically controlled emission and programmed time/temperature profiles. The other experimental data were as described in reference 13.

REFERENCES

1. Beckey HD, and Schulten H-R. Field desorption mass spectrometry. Angew Chem [Engl] 14:403-415, 1975.
2. Schulten H-R, and Beckey HD. High resolution field desorption mass spectrometry. I: Nucleosides and nucleotides. Org Mass Spectrom 7:861-867, 1973.
3. Schulten H-R, and Games DE. High resolution field desorption mass spectrometry. II. Glycosides. Biomed Mass Spectrom 1:120-123, 1974.
4. Schulten H-R, and Beckey HD. Field desorption mass spectrometry of anticancer drugs and their metabolites. *In* Proceedings of the 20th Annual Conference on Mass Spectrometry and Allied Topics. Dallas, June 4-9, 1972. Conference Proceedings, G-3.
5. Schulten H-R, Prinz H, Beckey HD, et al. Field desorption mass spectrometry: a new method for the identification of pesticides and their metabolites. Chemosphere 2:23-30, 1973.
6. Schulten H-R, and Beckey HD. Field desorption mass spectrometry of pesticides and their metabolites. J Agric Food Chem 21:372-375, 1973.
7. Schulten H-R, and Röllgen FW. On field desorption mass spectrometry of salts. Org Mass Spectrom 10:649-659, 1975 [and other references cited].
8. Schulten H-R, Beckey HD, Boerboom AJH, et al. Pyrolysis field desorption mass spectrometry of deoxyribonucleic acid. Anal Chem 45:2358-2362, 1973.
9. Schulten H-R, and Beckey HD. Field desorption mass spectrometry with high temperature activated emitters. Org Mass Spectrom 6:885-895, 1972.
10. Schulten H-R, Beckey HD, Eckhardt G, et al. Comparison of electron impact and field desorption mass spectra. I: Substituted sultams. Tetrahedron 29:3861-3868, 1973.
11. Brent DA, de Miranda P, and Schulten H-R. Field desorption mass spectrometry of azathioprine and its metabolites. J Pharm Sci 63:1370-1375, 1974.
12. Schulten H-R. Application of field desorption mass spectrometry to problems in the analysis of environmental chemicals. *In* New Methods in Environmental Chemistry and Toxicology (Coulston F, Korte F, and Goto M, eds). Tokyo, International Academic Printing Co, 1973, pp 31-42.
13. ____. Field desorption mass spectrometry of metabolites of the anticancer drug cyclophosphamide. Biomed Mass Spectrom 1:223-230, 1974.
14. Röllgen FW. Field ionization of organic molecules in fast reactions on surfaces. *In* Physical Chemistry of Fast Reactions (Hayward DO, ed). London, Plenum Publishing Corp, Ltd. In press, 1976, vol 2.
15. Schulten H-R, and Röllgen FW. Application of field desorption mass spectrometry in inorganic chemistry: salts. Angew Chem [Engl] 87:544-545, 1975.
16. Przybylski M, Ringsdorf H, Voelcker G, et al. Characterization of cyclophosphamide (NSC-26271) metabolites and related derivatives by field-desorption and electron-impact mass spectrometry. Cancer Treat Rep 60:509-516, 1976.
17. Cox PJ, Farmer PB, Foster AB, et al. The use of deuterated analogs in qualitative and quantitative investigations of the metabolism of cyclophosphamide (NSC-26271). Cancer Treat Rep 60:483-491, 1976.

Characterization of Cyclophosphamide (NSC-26271) Metabolites and Related Derivatives by Field-Desorption and Electron-Impact Mass Spectrometry[1,2]

M. Przybylski,[3,*] H. Ringsdorf,[3] G. Voelcker,[4] U. Draeger,[4] G. Peter,[4] and H.-J. Hohorst[4,5]

SUMMARY

Several important metabolites of cyclophosphamide (CP), such as 4-hydroxycyclophosphamide and phosphoramide mustard, and some 4-alkyl(aryl)thio derivatives have been investigated by field-desorption and electron-impact mass spectrometry. The structural identification of synthetic compounds and of derivatives, isolated by thin-layer chromatography in vitro, was possible since complementary information can be obtained using the two ionization techniques. Whereas only the field-desorption mass spectra showed more abundant molecular ions, the electron-impact technique revealed a characteristic fragmentation pattern for most of the compounds studied. Examples are given which demonstrate the existence of stereoisomers of CP derivatives using thin-layer chromatography and field-desorption mass spectrometry. The results indicate that field-desorption mass spectrometry is also a particularly appropriate method for the characterization of unstable CP metabolites in vivo.

[Cancer Treat Rep 60:509-516, 1976]

The metabolic activation of the anticancer agent cyclophosphamide (CP) has recently been the subject of detailed investigations (1-3). 4-Hydroxycyclophosphamide (4-hydroxy-CP), formed in the primary oxidation step by "mixed-function" oxidases in the liver, and its acyclic tautomer, aldophosphamide, were shown to be the key intermediates in the metabolism of this drug (4). The isolation and identification of these and other important metabolites presented several difficulties which are caused mainly by the instability of the compounds, particularly the primary oxidation products.

Mass spectrometry has been extensively applied to the characterization of CP and its metabolites (5-8). The unequivocal structural assignment of these compounds is, however, often complicated when electron-impact (EI) ionization is used. EI mass spectra yield, in most cases, only very weak or even no molecular ions, although diagnostic fragments can be obtained. Degradation of the compounds occurs readily when EI ionization is used, both by thermal decomposition, particularly at higher ion source temperatures, and by fragmentation. More recently, field-desorption (FD) mass spectrometry (9) has been used for the characterization of metabolites of CP, since this mild ionization technique obviates the evaporation of the sample prior to its ionization, and less excess energy is transferred to the molecule by its high field-induced ionization (10). Thus, intense ions of the intact molecules can be obtained (11).

In the present study, a comparative investigation of some of the more labile CP metabolites by FD and EI mass spectrometry is reported, together with mass spectra of model compounds which were useful in the study of the chemical reactivity of CP after its primary hydroxylation (12,13). More-

[1]Supported by a research program granted by the Bundesministerium für Forschung und Technologie.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]Institute of Organic Chemistry, University of Mainz, West Germany.

[4]Gustav Embden-Center of Biological Chemistry, Department of Cell Chemistry, University of Frankfurt, West Germany.

[5]We wish to thank Dr. H. R. Schulten, University of Bonn, West Germany, for stimulating discussions concerning field desorption mass spectral techniques. The technical assistance of M. Berner and W. Pracht is gratefully acknowledged. We are indebted to Asta-Werke AG, Bielefeld, West Germany, for supplying samples of 4-hydroxycyclophosphamide, phosphoramide mustard, and aldophosphamide 1,3-propanediol acetal.

Reprint requests to: Dr. M. Przybylski, Institute of Organic Chemistry, University of Mainz, D-65 Mainz, West Germany.

over, 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP) (14) and products of several in vitro studies on the reaction of 4-hydroxy-CP with mercaptans, which are part of a wider study on the activation of CP, have been characterized by mass spectrometry. A particular aim of this study was the identification of stereoisomers, which frequently complicate the assay of CP derivatives by chromatographic procedures (12,13,15).

EXPERIMENTAL PROCEDURES

In vitro and in vivo experiments, chromatographic separations, and preparation methods have been described elsewhere (12,13,15). EI mass spectra were recorded with a double-focusing spectrometer Varian MAT CH 711 at a resolution of 1000 (10%). All measurements were carried out at 70 eV, with an ion source temperature of 60°C-70°C, an 8 kV ion accelerating voltage, and a 1.8 kV electron multiplier voltage. The ion source pressure was always $<2 \times 10^{-6}$ torr. The samples were introduced in gold probes, by direct insertion. Metabolites (40-50 nmol) isolated by thin-layer chromatography (TLC) were eluted with 100 μl acetone or CH_2Cl_2 and, after centrifugation, were slightly concentrated. A 10-15 μl aliquot was, after removal of solvent, introduced into the ion source of the mass spectrometer. For detection of remaining impurities, reference spectra after elution of the TLC coating material have been determined.

The FD mass spectra were obtained with the same instrument equipped with a combined EI/FI/FD ion source. The experimental conditions were as follows: 800 (10%) resolution, 11 kV potential difference between the field emitter anode/cathode, 70°C ion source temperature, 90°C-100°C ion extraction heater temperature, and 2.5 kV electron multiplier voltage. An accessory high-voltage device (Varian) was used for the preparation of the activated 10 μm tungsten field emitters (16). The concentrations of metabolites isolated by TLC and the preparation procedures for FD measurements were similar to those for the EI spectra. Due to the lower sensitivity in the FD mode, the total eluate was concentrated to 20 μl and used directly for the FD measurements. The solvents used are given with the spectra. The samples were introduced by direct insertion, using the "emitter dipping technique" (16). The temperature of the emitter anode necessary to obtain a sufficiently intense ion current was obtained by slowly raising the heating current from 0 to 15-20 milliamperes, and several spectra were recorded within this range to determine the temperature producing the most abundant molecular ions (10). The heating currents given on the spectra are referred to this "optimal anode temperature," which cannot yet be measured exactly in the FD mode.

The registration of the EI spectra was carried out by an "on-line" 12 k computer 620i (Varian) on magnetic tape. High-boiling perfluorkerosine was used as a reference compound. The FD spectra were recorded both by computer and by an Oscillofil ultraviolet recorder (Siemens).

RESULTS AND DISCUSSION

Metabolites of CP

Several attempts were made to obtain sufficiently characteristic EI mass spectra of 4-hydroxy-CP after its thin-layer chromatographic isolation in vitro, before an FD ion source was available in our laboratory. In spite of careful techniques applied to the transfer of the compound from a solution by direct insertion into the ion source of the mass spectrometer, partial decomposition in the ion source could not be prevented.

Figure 1 (*a* and *b*) shows the comparison of the EI spectra of the synthetic 4-hydroxy-CP with a product eluted after separation by TLC and believed to be the diastereomer. Both compounds revealed an essentially similar mass spectral pattern but no complete identity could be determined due to residual impurities from the TLC plate. (Peaks due to remaining TLC material were identified by reference spectra of the coating material and are indicated in fig 1[*b*] as dashed lines.)

The molecular ion (m/e 276) could not be detected, but the appearance of a small peak at m/e 277 suggests the identity of the two compounds, although the formation of an $(M + 1)^+$ ion is very unusual in EI mass spectrometry. Further evidence for the existence of the two diastereomeric forms arises from the fragments at m/e 227 (4% and 7% relative intensity respectively) attributable to the loss of a CH_2Cl radical from the mustard moiety of the molecule. This fragmentation pathway was frequently found capable of distinguishing the structures of CP derivatives (1,5). Moreover, nearly all other more abundant fragments are present in both spectra: m/e 184, 141 (mustard group, 4% and 7%), 92 (one Cl, 100% and 68%), 84 (24% and 46%), 63 (one Cl, 29% and 27%), and 56 (39% and 100%).

A clear structural proof of 4-hydroxy-CP is obtained by the FD spectrum shown in figure 1(*c*). At 20 milliamperes emitter heating current, representing the "optimal anode temperature"[6] for this

[6]The "optimal anode temperature" is defined as the emitter temperature producing the greatest relative abundance of the molecular ion (10).

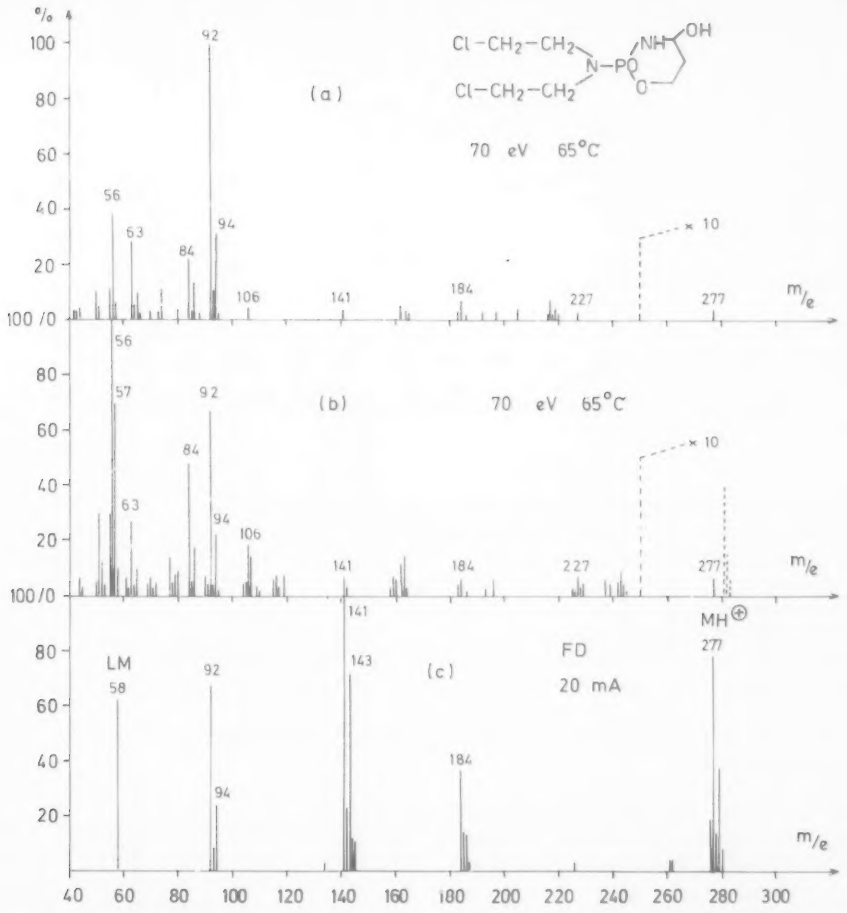


FIGURE 1.—EI and FD mass spectra of 4-hydroxy-CP. (a) EI spectrum of a synthetic product; (b) EI spectrum of the presumed diastereomer isolated by TLC; (c) FD mass spectrum of 4-hydroxy-CP; acetone solvent.

compound, an $(M + 1)^+$ ion (MH^+) of about 80% was found. At this temperature, fragments formed by cleavage at the mustard moiety were also present (m/e 141, 92). By FD ionization, the appearance of an $(M + 1)^+$ peak is frequently observed and is caused by protonation of the molecule on the emitter surface.

Evidence for the formation of diastereomers was also observed in vivo (15) when 4-hydroxy-CP was, after treatment with ethanol, isolated from rat serum by TLC and investigated by EI mass spectrometry. The resulting spectra were substantially identical with those previously reported for 4-ethoxycyclophosphamide (1), thus showing a molecular ion at m/e 304/306 and prominent peaks at m/e 259, 255/257, and 209/211, which are clearly attributed to the ethoxy derivative.

No evidence for an acetalization reaction of 4-hydroxy-CP was found when TLC plates coated with 1,3-propanediol were used for its isolation. The FD mass spectrum of the authentic propanediol acetal (fig 2) contained, at 15-20 milliamperes, almost exclusively the molecular ion at m/e 334/336. This peak was never observed for any compounds isolated by TLC. Thus, the possibility of an acetalization via the tautomeric aldophosphamide on the TLC plate, as indicated in the reaction of 4-hydroxy-CP with 1,3-propanedithiol (12), might be excluded.

Preliminary studies have been carried out on the

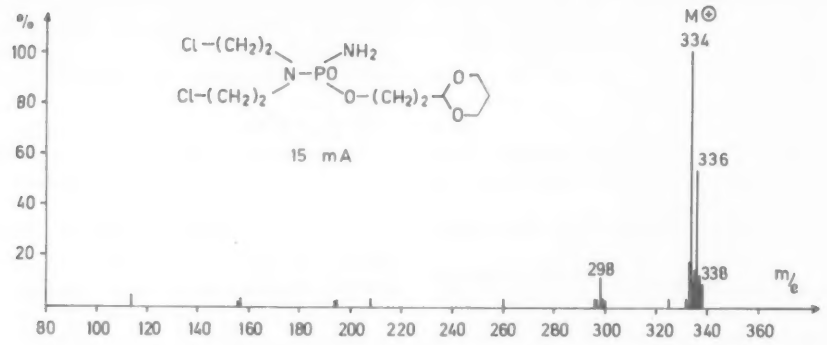


FIGURE 2.—FD mass spectrum of aldophosphamide 1,3-propanediol acetal; acetone solvent.

characterization of aldophosphamide by FD mass spectrometry. However, no molecular ion due to aldophosphamide could be measured in these experiments. Indications for the decomposition of this highly unstable compound were observed after its isolation by TLC, indicating that especially careful preparation requirements are needed for the FD measurement. Besides the presence of peaks attributable to *nor*-nitrogen mustard, the spectra obtained gave no evidence for any other metabolite or degradation product of CP.

Figure 3 shows the EI and FD mass spectra of phosphoramide mustard [*N,N*-bis(2-chloroethyl)-phosphorodiamidic acid]. This important metabolite (8) of CP (probably the ultimate cytotoxic metabolite) has been characterized, after derivatization, by EI (7) and FD mass spectrometry as the cyclohexylammonium salt (11). However, comparing the EI and FD spectra, a clear structural identification of the free acid is possible. As was expected, the EI spectra show only traces of ions around the molecular weight of 220 (m/e 219, $[M-H]^+$, 0.4%), but show a small peak at m/e 171 ($M-CH_2Cl$) and the typical fragmentation pattern of the mustard group (m/e 92, 63/65, 56). The complementary structural information is obtained by the FD spectrum where the $(MH)^+$ peak (m/e 221/223) arises as base peak. The *nor*-nitrogen mustard fragment m/e 141 is the only more abundant fragment and suggests a thermally induced fission of the mustard P-N linkage.

Activated Derivatives of CP

The low chemical stability of the activated CP derivatives is illustrated by both the EI and FD mass spectra of 4-hydroperoxy-CP (14) (fig 4). At a

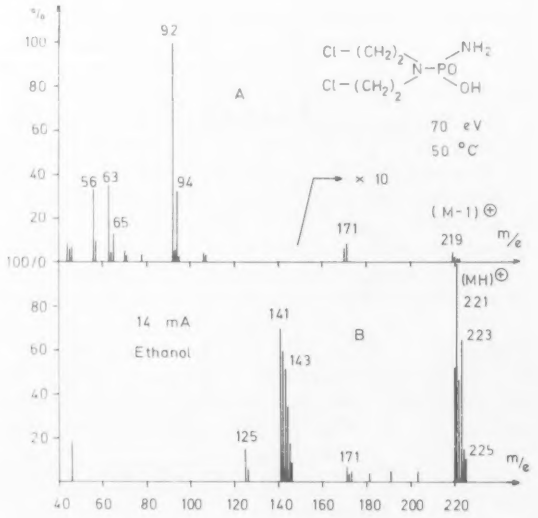


FIGURE 3.—EI (A) and FD (B) mass spectra of phosphoramide mustard.

temperature of the emitter anode carefully regulated to obtain the most intense molecular ion possible, an $(MH)^+$ ion of 20%, with a multiplicity of intense degradation products, was found in the FD spectra. Among other fragmentation pathways, the loss of H_2O and H_2O_2 yielded 4-ketocyclophosphamide (m/e 274) and the CP fragment m/e 258, respectively, as diagnostic fragments. In the EI spectra (fig 4[A]) the molecular ion is missing and only a small signal at m/e 259 is found, due to the loss of a hydroperoxy radical from the molecule. Further, most abundant fragment ions are unspecific with regard to the structural determination of this compound.

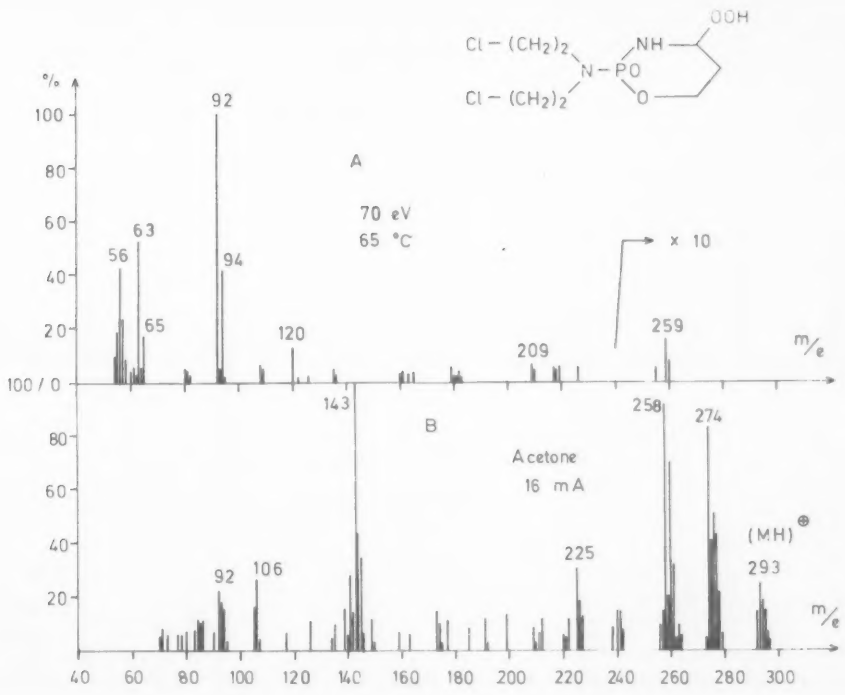


FIGURE 4.—EI (A) and FD (B) mass spectra of 4-hydroperoxy-CP.

4-Ethylthiocyclophosphamide (4-ethylthio-CP) was the first among a series of the latent activated sulfur-containing derivatives obtained by reaction of 4-hydroxy-CP with different mercaptans (12). The attempt to characterize this highly unstable compound by its EI mass spectrum was unsuccessful. The EI spectrum shown in figure 5(*a*) contained only a small peak (m/e 259) due to CP and the fragments attributable to any particular CP derivative, which did not allow a structural identification on this basis.

Subsequently, FD mass spectra were recorded which showed the greater use of this mild ionization technique. Figure 5(*b* and *c*) shows the FD spectra of two reaction products of 4-hydroxy-CP and ethylmercaptan, having different TLC mobilities (12). Both compounds show a molecular ion at m/e 320/322 with comparable intensities (19% and 22% respectively). The complete identity of the spectra gives clear evidence for the two diastereomers of 4-ethylthio-CP. Moreover, due to the high intensity of the CP fragment m/e 259 $(M\text{--}S\text{-}C_2H_5)^+$, any structure other than the cyclic structure for the products seems unlikely.

These findings were supported by mass spectral studies of the reaction product obtained from 4-hydroxy-CP and tert.-butylmercaptan. The FD mass spectrum of 4-tert.-butylthiocyclophosphamide shows the same pattern as the ethylthio derivative, containing the molecular ion at m/e 348/350 (27%) and the base peak at m/e 259/261 (fig 6 [B]). A peak at m/e 90 due to the tert.-butylmercaptan is also observed. Because of the higher stability of this compound, the EI spectrum (fig 6[A]) was also informative in the determination of the structure. Besides a small M^+ peak detectable at m/e 348/350, nearly all the more abundant fragment ions can be interpreted and follow the typical pathways observed for CP derivatives (fig 7).

An even higher stability and a more informative EI mass spectrum was expected for 4-benzylthiocyclophosphamide (4-benzylthio-CP). Investigation of the reaction of 4-hydroxy-CP and benzylmercaptan by TLC again showed two products, suggesting the formation of diastereomers. Surprisingly, their EI spectra gave neither a molecular ion nor any specific fragments in the higher mass range. High intensity ions for benzylmercaptan (m/e 124)

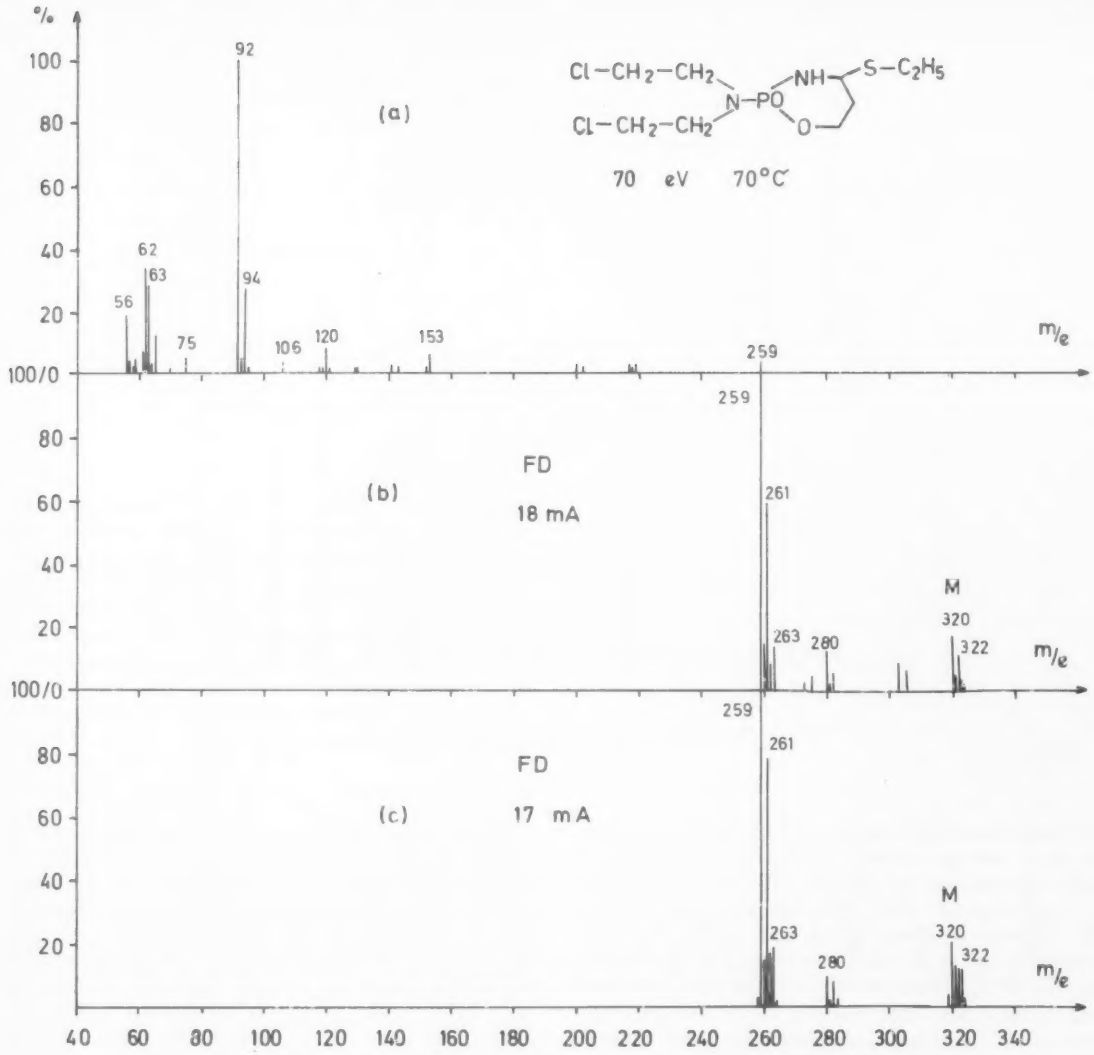


FIGURE 5.—Mass spectra of 4-ethylthio-CP. (a) EI spectrum of the product obtained from 4-hydroxy-CP and ethylmercaptan; (b) FD spectrum of this compound; (c) FD spectrum of the diastereomeric product isolated by TLC; acetone solvent.

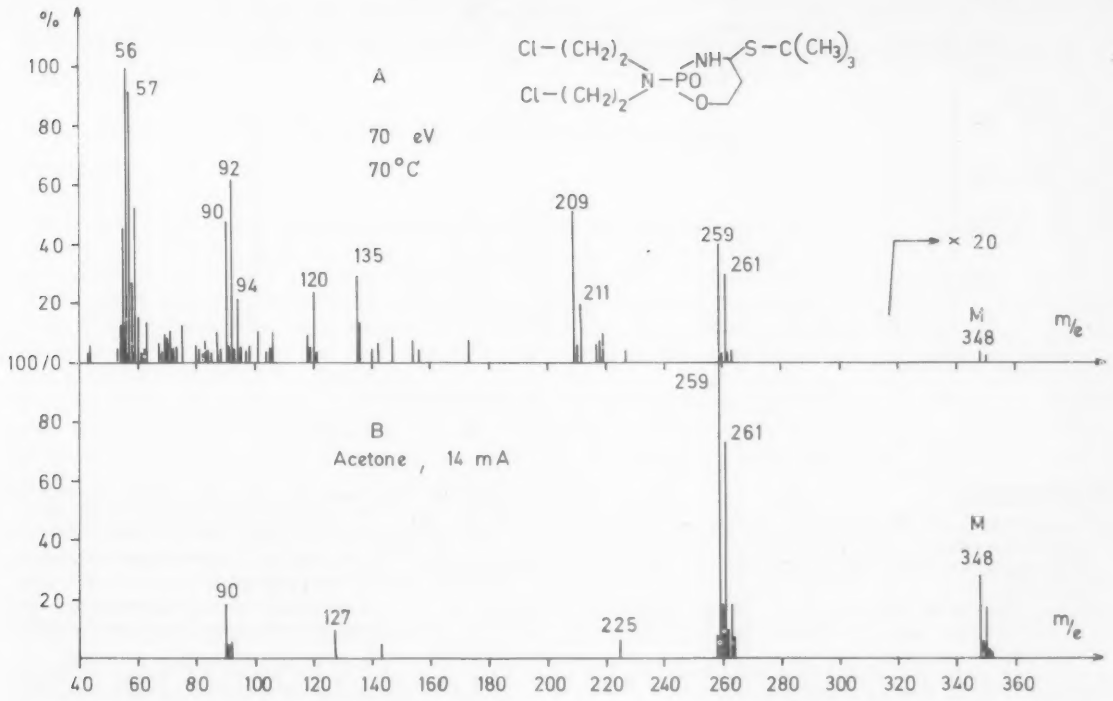


FIGURE 6.—EI (A) and FD (B) mass spectra of 4-tert.-butylthiocyclophosphamide.

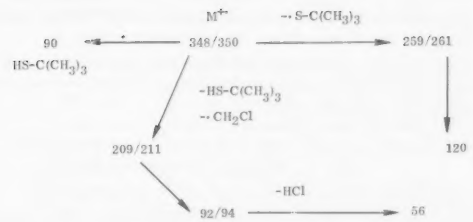


FIGURE 7.—Fragmentation pathways of 4-tert.-butylthiocyclophosphamide.

and C_6H_5-CH_2^+ (m/e 91) were found. However, the FD spectrum (fig 8) of the more mobile "fast" compound showed the $(M + 1)^+$ peak at m/e 383 (100%) and the CP fragment at m/e 259/261 (18%). Other prominent ions at m/e 336 and 278 could not be interpreted and might be due to impurities. Remaining impurities and/or decomposition products were also found in the FD mass spectrum of the "slow" product, which gave a rather intense signal at m/e 383 appropriate to the diastereomer, but with even higher intensities, peaks occurred above the molecular weight of 4-benzylthio-CP. Hence, the possibility of the formation of by-products via the tautomeric aldophosphamide might not be excluded, and further mass spectral studies to elucidate the reaction pathways of 4-hydroxy-CP with nucleophilic reagents of this type are necessary.

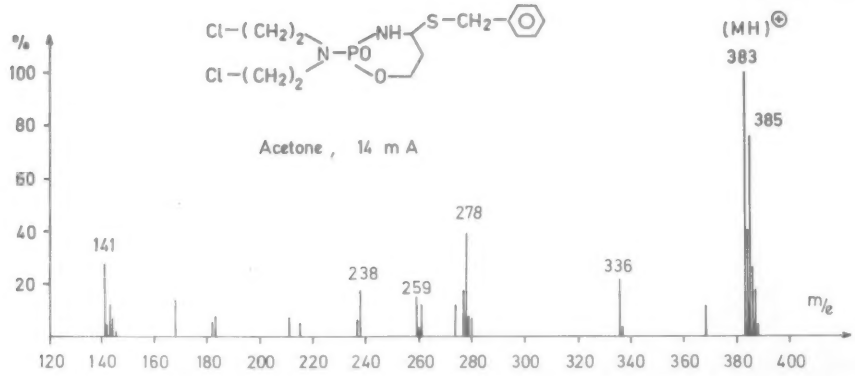


FIGURE 8.—FD mass spectrum of 4-benzylthio-CP.

REFERENCES

1. CONNORS TA, COX PJ, FARMER PB, ET AL. Some studies on the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-120, 1974.
2. STRUCK RF, KIRK MC, MELLETT LB, ET AL. Urinary metabolites of the antitumor agent cyclophosphamide. Mol Pharmacol 7:519-529, 1971.
3. HILL DL. A Review of Cyclophosphamide. Springfield, Ill, C. C. Thomas, 1975, pp 25-59 [and references therein].
4. VOELCKER G, DRAEGER U, PETER G, ET AL. Studies on the spontaneous decomposition of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide by means of thin layer chromatography. Arzneim Forsch 24:1172-1176, 1974.
5. CONNORS TA, COX PJ, FARMER PB, ET AL. Observations on the mechanisms of hydroxylation of cyclophosphamide by rat liver microsomes: the metabolism of cyclophosphamide-4-d_2. Biomed Mass Spectrom 1:130-136, 1974.
6. COX PJ, FARMER PB, and JARMAN M. The microsomal metabolism of some analogues of cyclophosphamide: 4-methylcyclophosphamide and 6-methylcyclophosphamide. Biochem Pharmacol 24:599-606, 1975.
7. COLVIN M, PADGETT CA, and FENSELAU C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
8. STRUCK RF, KIRK MC, WITT MH, ET AL. Isolation and mass spectral identification of blood metabolites of cyclophosphamide: evidence for phosphoramide mustard as the biological active metabolite. Biomed Mass Spectrom 2:46-52, 1975.
9. BECKEY HD. Field desorption mass spectrometry. A technique for the study of thermally unstable substances of low volatility. Int J Mass Spectrom Ion Phys 2:500-509, 1969.
10. BECKEY HD, and SCHULTEN HR. Felddesorptions-Massenspektrometrie. Angew Chem 87:425-438, 1975.
11. SCHULTEN HR. Field desorption mass spectrometry of the anticancer drug cyclophosphamide. Biomed Mass Spectrom 1:223-230, 1974.
12. PETER G, WAGNER TH, and HOHORST H-J. Studies on 4-hydroperoxycyclophosphamide (NSC-181815): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. Cancer Treat Rep 60:429-435, 1976.
13. DRAEGER U, PETER G, and HOHORST H-J. Deactivation of cyclophosphamide (NSC-26271) metabolites by sulfhydryl compounds. Cancer Treat Rep 60:355-359, 1976.
14. TAKAMIZAWA A, MATSUMOTO S, IWATA T, ET AL. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
15. VOELCKER G, WAGNER TH, and HOHORST H-J. Identification and pharmacokinetics of cyclophosphamide (NSC-26271) metabolites in vivo. Cancer Treat Rep 60:415-422, 1976.
16. SCHULTEN HR, and BECKEY HD. Field desorption mass spectrometry with high temperature activated emitters. Org Mass Spectrom 6:885-895, 1972.

X-Ray Crystallographic Studies of Cyclophosphamide (NSC-26271) Derivatives[1,2]

Arthur Camerman,[3,*] H. Warren Smith,[3] and Norman Camerman[4]

SUMMARY

Molecular structures of 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP) and of two stereoisomers of 4-hydroperoxyisophosphamide (4-hydroperoxy-IP) have been determined by X-ray crystallography. All compounds, synthesized by Dr. A. Takamizawa by ozonolysis of appropriate open-chain precursors, demonstrate in vivo and in vitro cytotoxicity. The structure results show that for 4-hydroperoxy-CP and both isomers of 4-hydroperoxy-IP the 4-hydroperoxy group is situated axial to the ring. In 4-hydroperoxy-CP and the isomer of 4-hydroperoxy-IP which is the major product of the ozonolysis reaction, the phosphoryl oxygen atom is also axial to the ring and thus *cis* to the C_4-oxygen. In the 4-hydroperoxy-IP isomer which is the minor product of the ozonolysis, the phosphoryl oxygen is equatorial to the ring and *trans* to the C_4-oxygen. A scheme is presented to account for the interconversion of the two 4-hydroperoxy-IP configurations. Since a stereoisomer with a hydroperoxy or hydroxy group equatorial has not been isolated from the ozonolysis synthesis or from the Fenton oxidation of cyclophosphamide (CP), the configuration with C_4-oxygen axial which is found in 4-hydroperoxy-CP and both isomers of 4-hydroperoxy-IP is probably more stable and may be the configuration of 4-hydroxy compounds formed by the in vivo oxidation of CP, isophosphamide, and trophosphamide (TP). The molecular structure of TP has also been elucidated. Comparison of the conformations of the three chloroethyl alkylating groups of this molecule with those of the two chloroethyl groups of other CP derivatives indicates that these groups are highly flexible and do not assume one favored conformation.

[Cancer Treat Rep 60:517-522, 1976]

Since its introduction into clinical medicine, cyclophosphamide (CP) (fig 1, I) has become one of the most widely used agents in the treatment of many types of cancer. It has also become a focal point for two areas of basic research: (*a*) development of analogs in the search for improved CP-like antitumor agents, and (*b*) investigations into the metabolism and mechanisms of action of this type of alkylating agent. Progress in the first area has resulted in synthesis of isophosphamide (IP) (fig 1, II) and trophosphamide (TP) (fig 1, III), both potentially clinically useful drugs. Advances in the second area have been doubly rewarding; not only has our knowledge of the metabolism and mechanism of action of the CP type of drug been extended, but new "pre-activated" 4-hydroxy derivatives which may prove useful as new antitumor agents have also been synthesized. We have undertaken X-ray-crystal structure determinations of new CP-like drugs and of compounds related to products of CP metabolism. We hope that the molecular parameters elucidated in our studies will prove useful in the design of new drugs and in understanding steps in CP metabolic pathways. We present here the results of the crystal and molecular structure

[1]Supported by Public Health Service grant CA-15879 (National Cancer Institute [NCI]) and Research Career Development Award NS-70801 (Dr. A. Camerman) from the National Institutes of Health, Department of Health, Education, and Welfare, by Institutional Cancer grant IN-26 from the American Cancer Society, and by the Medical Research Council of Canada.

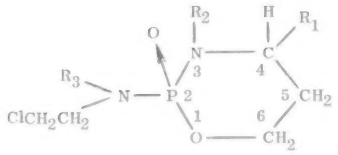
[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

4-Hydroperoxy-CP and 4-hydroperoxy-IP were supplied by Dr. Akira Takamizawa, Shionogi Research Laboratory, Shionogi and Co, Ltd. TP was supplied by the Drug Development Branch, Drug Research and Development Program, Division of Cancer Treatment, NCI.

[3]Departments of Medicine (Neurology) and Pharmacology, University of Washington, Seattle.

[4]Department of Biochemistry, University of Toronto, Canada.

*Reprint requests to: Dr. Arthur Camerman, Department of Medicine, University of Washington, Seattle, Wash 98195.



I. CP: $R_1 = R_2 = H$; $R_3 = CH_2CH_2Cl$

II. IP: $R_1 = R_3 = H$; $R_2 = CH_2CH_2Cl$

III. TP: $R_1 = H$; $R_2 = R_3 = CH_2CH_2Cl$

IV. 4-Hydroperoxy-CP: $R_1 = OOH$; $R_2 = H$; $R_3 = CH_2CH_2Cl$

V. 4-Hydroperoxy-IP: $R_1 = OOH$; $R_2 = CH_2CH_2Cl$; $R_3 = H$

FIGURE 1.—CP and CP derivatives.

determinations of 4-hydroperoxycyclophosphamide (4-hydroperoxy-CP) (fig 1, IV), of two stereoisomers of 4-hydroperoxyisophosphamide (4-hydroperoxy-IP) (fig 1, V), and of TP.

4-HYDROPEROXY-CP

It is becoming increasingly clear that the first step in the activation of CP (and its analogs IP and TP) is mono-oxidation by the mixed-function oxidases of liver microsomes to form the 4-hydroxy derivative. It had been thought that 4-hydroxycyclophosphamide (4-hydroxy-CP) was the active antitumor metabolite of CP but recent evidence (1,2) suggests that it undergoes decomposition to yield acrolein and phosphoramide mustard with the latter being the ultimate cytostatic CP metabolite. We have previously determined the crystal structure of one of the metabolic end products of CP, 4-ketocyclophosphamide (4-keto-CP) (3), but neither these results nor those for CP (4) yield information about the important configuration at C_4 of the hydroxylated antitumor species. Recently, Takamizawa et al (5) synthesized 4-hydroxy-CP and 4-hydroperoxy-CP. They found that both synthetic products exhibit pronounced in vitro and in vivo cytostatic activities and that 4-hydroperoxy-CP can readily be converted to 4-hydroxy-CP by chemical and biologic reduction. 4-Hydroxy-CP is highly unstable and is not amenable to crystallographic studies so we have chosen to determine the crystal and molecular structure of 4-hydroperoxy-CP in order to elucidate the configuration about C_4 in these synthetic CP derivatives. Since they exhibit high cytostatic activity, it is probable that these compounds will have the same C_4 configuration as the activated 4-hydroxy-CP metabolite.

The crystals of 4-hydroperoxy-CP supplied by Dr. Takamizawa are colorless plates which turn pale yellow and exhibit moderate decomposition in the X-ray beam. They are monoclinic, space group $P2_1/c$. Unit cell parameters for all four compounds reported here are listed in table 1.

Figure 2 is a stereoscopic drawing showing the three-dimensional molecular structure of 4-hydroperoxy-CP. The six-membered ring is in the chair conformation and the configuration about the phosphorus atom has the bis(chloroethyl)amine group equatorial and the phosphoryl oxygen atom axial, the same as that found in 4-keto-CP and in CP. The major difference between the inactive compounds and activated 4-hydroxy-CP and 4-hydroperoxy-CP is the hydroxy (or hydroperoxy)

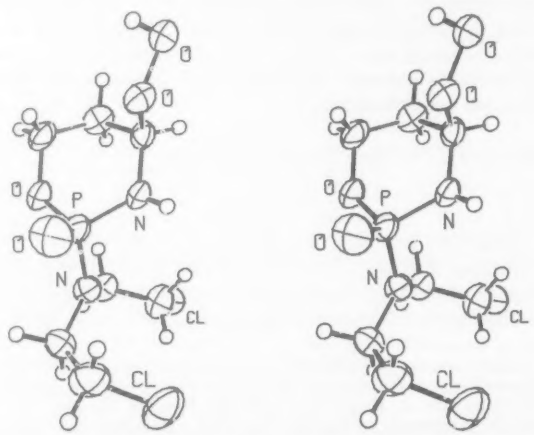


FIGURE 2.—Stereoscopic drawing of the molecular structure of 4-hydroperoxy-CP. The ellipsoids illustrate the thermal motion of the atoms. (To gain the three-dimensional benefit of the stereoscopic drawings, figs 2-5 should be viewed with the aid of a stereo viewer.)

TABLE 1.—Crystal data

Parameters	4-Hydroperoxy-CP	*cis*-4-Hydroperoxy-IP	*trans*-4-Hydroperoxy-IP	TP
a, Å	14.299	8.999	15.185	13.722
b, Å	7.706	8.743	10.345	13.929
c, Å	11.891	17.078	18.205	8.021
β, degrees	103.06	107.92	114.15	100.49

group at C_4. The significant finding in our structure determination of 4-hydroperoxy-CP is that this group is situated axial to the ring. It is thus *cis* to the phosphoryl oxygen and *trans* to the bis-(chloroethyl)amine group. The distance between the C_4-oxygen atom and the phosphoryl oxygen is 3.54 Angstroms (Å); the same distance would pertain for synthetic 4-hydroxy-CP.

The synthetic 4-hydroperoxy-CP prepared by Takamizawa et al (5) by ozonolysis of an appropriate open-chain compound is a racemic mixture of *d*- and *l*-isomers, both, of course, having the C_4-oxygen axial and *cis* to the phosphoryl oxygen. 4-Hydroperoxy-CP has also been produced by the Fenton oxidation of CP and was shown to be identical to synthetic 4-hydroperoxy-CP (6). In neither synthesis was an isomer with the C_4-oxygen in the equatorial position isolated. In addition, 4-peroxycyclophosphamide (4-peroxy-CP) has been produced from the Fenton oxidation of CP (6-8) and both halves of this dimer have the C_4-oxygen in the axial configuration (9). Since all of the synthetically prepared compounds (4-hydroxy-CP, 4-hydroperoxy-CP, and 4-peroxy-CP) are essentially equivalent in biologic behavior to the active species of CP, and all possess the activating C_4-hydroxyl oxygen in the axial position, it seems reasonable to suggest that this configuration is the most stable one for the C_4-hydroxylated CP compounds and may be the configuration of the 4-hydroxy-CP produced in in vivo activation of CP. Support for this latter suggestion comes from the reported (7) in vivo occurrence of 4-peroxy-CP identical to that obtained from the chemical reduction of synthetic 4-hydroperoxy-CP (8), although that result has not yet been duplicated.[5]

4-HYDROPEROXY-IP

In a manner analogous to their synthesis of 4-hydroperoxy-CP, Takamizawa et al (10) have prepared 4-hydroperoxy-IP. As is true for 4-hydroperoxy-CP, 4-hydroperoxy-IP is readily convertible to the corresponding 4-hydroxyisophosphamide (4-hydroxy-IP) which like 4-hydroxy-CP is an activated metabolite (of IP) and is unstable compared to the hydroperoxy compound. Recently it has been found that 4-hydroperoxy-IP readily forms an acid-catalyzed equilibrium mixture with an epimer which is identical to a minor side-product of the ozonolysis synthesis procedure.[6] Both epimers display in vitro and in vivo cytotoxic activity.

The reasons for investigating the crystal structure of 4-hydroperoxy-CP discussed above pertain equally to 4-hydroperoxy-IP. The isolation of two isomers lends even more importance to their molecular structure determinations. Since both isomers are cytostatically active compounds, similarities and differences between the two 4-hydroperoxy-IP structures and between them and 4-hydroperoxy-CP will be highly significant in determining which configurational features might be necessary for antitumor activity and also in helping to further understand the pathways of IP activation and metabolism.

The major ozonolysis product which we have named *cis*-4-hydroperoxy-IP, was recrystallized from 95% ethanol. It exists in two crystalline polymorphic forms: triclinic, space group $P\bar{1}$, and monoclinic, space group $P2_1/c$. We have determined the structure of the latter polymorph; it is to be expected that both crystal forms would yield the same molecular conformation with possibly minor changes in the conformation of the chloroethyl chains.

The molecular structure of *cis*-4-hydroperoxy-IP is shown in figure 3. The configurations at the phosphorus atom and at C_4 are the same as found in 4-hydroperoxy-CP: phosphoryl oxygen atom and C_4-hydroperoxy group in the axial positions and the chloroethylamine group equatorial. (Because the phosphoryl and C_4-oxygen atoms are *cis* to one another, we have arbitrarily labeled this compound *cis*-4-hydroperoxy-IP.) The distance between the phosphoryl and C_4-oxygen atoms in *cis*-4-hydroperoxy-IP is 3.76 Å, slightly larger than the comparable distance in 4-hydroperoxy-CP. It has been observed that the N_3-CH_2CH_2Cl and C_4-hydroperoxy groups of *cis*-4-hydroperoxy-IP are readily cyclized on treatment with alkali to form a bicyclic peroxide.[6] It can be easily seen from our results why this is so. Figure 3 shows that the N_3 atom of *cis*-4-hydroperoxy-IP is trigonal with the first methylene group in the P_2-N_3-C_4 plane. The second methylene group points up from the first one, with the CH_2-CH_2 bond parallel to the C_4-oxygen bond. Thus, one simple bond rotation would bring the second methylene carbon and peroxy oxygen atoms into close proximity for elimination of HCl and formation of the bicyclic peroxide.

The minor side-product of the ozonolysis synthesis of 4-hydroperoxy-IP (and also obtainable by dissolving *cis*-4-hydroperoxy-IP in acid solution) is an isomer of 4-hydroperoxy-IP which we have named *trans*-4-hydroperoxy-IP; the label, as in the previous 4-hydroperoxy-IP isomer, describes the relationship between the phosphoryl and C_4-oxygen atoms.

Crystals of *trans*-4-hydroperoxy-IP are monoclinic, space group $P2_1/c$, with two molecules form-

[5]Struck RF. Personal communication.

[6]Takamizawa A. Personal communication.

ing the asymmetric unit instead of one as found for the other compounds studied.

Figure 4 shows the molecular structures of the two crystallographically independent molecules of *trans*-4-hydroperoxy-IP. The only conformational dissimilarity between them is the orientation of the exocyclic chloroethylamine group which differs considerably from one molecule to the other. In all other respects they appear identical. Structural features to note are: (*a*) the

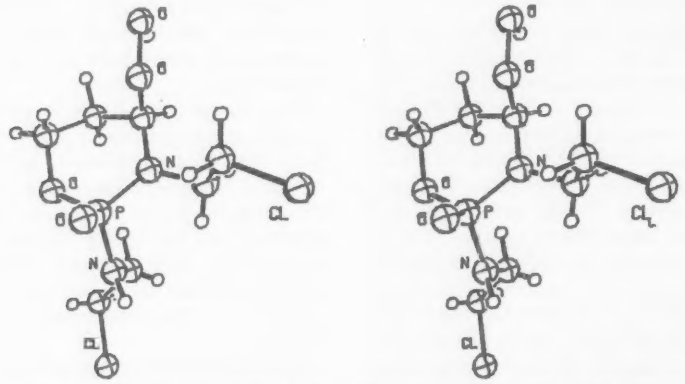


FIGURE 3.—Stereoscopic drawing of the molecular structure of the 4-hydroperoxy-IP stereoisomer with the phosphoryl oxygen atom in the axial position.

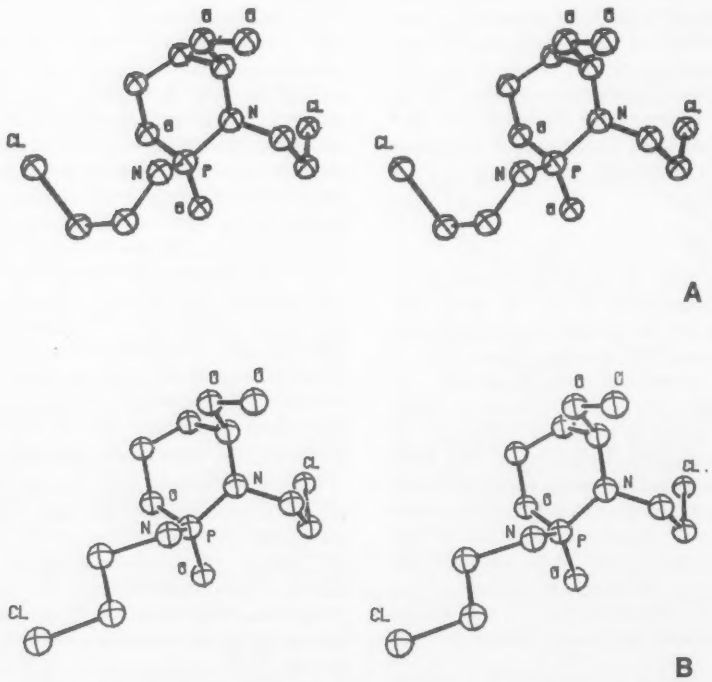


FIGURE 4.—Stereoscopic drawing of the molecular structure of the 4-hydroperoxy-IP stereoisomer with the phosphoryl oxygen atom in the equatorial position; A = molecule 1; B = molecule 2. Hydrogen atoms are not shown.

conformation of the ring is identical to 4-hydroperoxy-CP and *cis*-4-hydroperoxy-IP; (*b*) the configuration at C_4 is α-axial hydroperoxy group, exactly as found in 4-hydroperoxy-CP and *cis*-4-hydroperoxy-IP; and (*c*) the configuration at the phosphorus atom is phosphoryl oxygen atom in the equatorial position and chloroethylamine group axial, the opposite configuration to that found for the other molecules studied. Thus, even though two stereoisomers of 4-hydroperoxy-IP can be isolated; the significant finding from our studies is that they both maintain the same configuration at C_4, the position at which hydroxylation changes the inactive CP compounds to activated species and that configuration is hydroperoxy (or hydroxy) group axial to the ring. The distance between the axial C_4-hydroxyl oxygen atom and the equatorial phosphoryl oxygen atom is 4.73 Å in both *trans*-4-hydroperoxy-IP molecules.

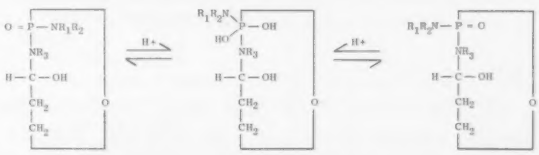
Since isomers of 4-hydroperoxy-CP (or 4-hydroxy-CP) and 4-hydroperoxy-IP (or 4-hydroxy-IP) with the C_4-hydroperoxy (or hydroxy) group equatorial to the ring do not seem to result from either the ozonolysis syntheses of Takamizawa et al or from the Fenton oxidation of CP or its analogs, it is not known whether they would exhibit cytostatic activity or whether they could be degraded to acrolein and phosphoramide mustard. In the absence of evidence for the existence of such isomers, it is reasonable to suggest that the configuration found in these structures (4-hydroxy group in the axial position) be maintained in the design of new pre-activated CP antitumor drugs.

Since both isomers of 4-hydroperoxy-IP display cytotoxicity, it is obvious that the configuration at phosphorus is not a factor in determining the biologic activity of these compounds. The inversion of configuration at the phosphorus atom does however appear to affect the conformation of the N_3-chloroethyl group. In *cis*-4-hydroperoxy-IP and in TP (see below) where the phosphoryl oxygen atom is axial and the chloroethylamine group is equatorial, the N_3-chloroethyl group has its CH_2-CH_2 bond parallel to C_4-O. In *trans*-4-hydroperoxy-IP, which has the opposite configuration at the phosphorus atom, the comparable CH_2-CH_2 and C_4-O bonds are antiparallel. We might speculate from this result that it would be slightly more difficult to form the alkali-catalyzed bicyclic peroxide from *trans*-4-hydroperoxy-IP than from *cis*-4-hydroperoxy-IP.

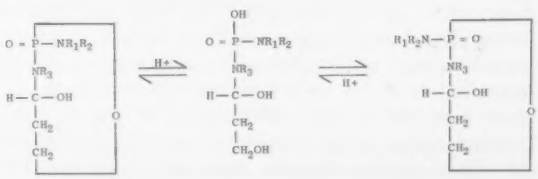
Connors et al (2) have isolated two ethyl derivatives of 4-hydroxy-CP from the in vivo metabolism of CP. They have suggested that the two metabolic products are stereoisomers of 4-hydroxy-CP differing by their configuration at C_4; one metabolite would contain the 4-hydroxy oxygen atom in the axial position and the other in the equatorial position. On the basis of our structural results with *cis*-4-hydroperoxy-IP and *trans*-4-hydroperoxy-IP we feel that it is much more likely that the two compounds both have the 4-hydroxy oxygen atom in the axial position but differ from one another in the configuration at the phosphorus atom. Added evidence for this point of view is that when they put one of their metabolites in acid solution, Connors et al obtained an equilibrium mixture of both isomers. This is exactly the finding with synthetic *cis*- and *trans*-4-hydroperoxy-IP.

It is not difficult to envision a mechanism of action for the acid-catalyzed interconversion of *cis*- and *trans*-4-hydroperoxy-IP (or the comparable hydroperoxy or hydroxy isomers of CP) with the retention of configuration at C_4.

Protonation of the phosphoryl oxygen atom followed by nucleophilic attack by a water molecule would lead to a dihydroxylated, penta-coordinate configuration at the phosphorus. Either stereoisomer could then be obtained depending on which of the chemically equivalent hydroxyl groups is removed in the subsequent dehydration.



A second, but less likely, mechanism which could occur via protonation of the ester oxygen could lead to a ring-opened intermediate which would be short-lived and would recyclize with resultant random configuration at the phosphorus but with no change in configuration at C_4.



The second scheme is somewhat analogous to the well-known mechanism for mutarotation of sugars.

It has not escaped our attention that this postulated mechanism, with the existence of the ring-opened intermediate, provides an alternate pathway for decomposition of 4-hydroxy-CP to acrolein and phosphoramide mustard other than through an aldophosphamide intermediate. If scission of the N_3-C_4 bond occurs to this ring-opened form of CP (possibly via an aldolase enzyme), with coincident aldehyde formation, the products would be phos-

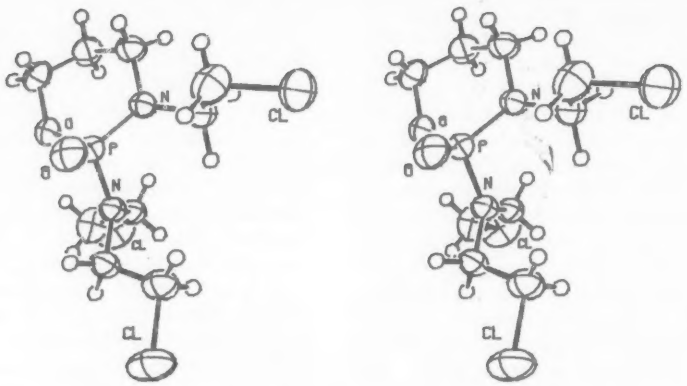


FIGURE 5.—Stereoscopic drawing of the molecular structure of TP. Ellipsoids illustrate the thermal motion of the atoms in the crystal.

phoramide mustard and $CH_2(OH)CH_2CHO$. The latter would immediately undergo dehydration to form acrolein. It is also of interest to note that the ring-opened intermediate is the 4-hydroxyl derivative of cytotoxyl alcohol and thus would be expected to be a potent cytotoxic agent itself.

TP

TP, the analog of CP with three *N*-chloroethyl groups instead of two (as for CP and IP), has shown potential as a clinically useful drug against a number of types of cancer. This compound was crystallized from a mixture of ether and heptane. Like the other CP derivatives, TP crystallizes in the monoclinic space group $P2_1/c$.

Figure 5 is a stereoscopic drawing of the molecular structure of TP. The ring is in the chair conformation with the configuration at the phosphorus being phosphoryl oxygen axial and bis(chloroethyl)-amine group equatorial. The chloroethyl group attached to N_3 is in a conformation which is almost identical to the conformation of the N_3-chloroethyl group of the *cis* isomer of 4-hydroperoxy-IP. This again suggests that the conformation adopted by this group depends heavily on the configuration at the phosphorus atom. Comparison of the conformations of the chloroethyl alkylating groups of TP with those found in 4-keto-CP, CP, 4-hydroperoxy-CP, and the two 4-hydroperoxy-IP isomers indicates that the exocyclic mustard groups, in particular, are very flexible and do not assume one favored conformation. It is likely that the important steric requirement for the chloroethyl chains is the ability for their reactive ends to be far enough apart to be able to alkylate separated bases on the DNA helices.

REFERENCES

1. COLVIN M, PADGETT CA, and FENSELAU C. A biologically active metabolite of cyclophosphamide. Cancer Res 33:915-918, 1973.
2. CONNORS TA, COX PJ, FARMER PB, ET AL. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem Pharmacol 23:115-129, 1974.
3. CAMERMAN N, and CAMERMAN A. Cyclophosphamide structure: molecular structure of 4-ketocyclophosphamide. J Am Chem Soc 95:5038-5041, 1973.
4. GARCIA-BLANCO S, and PERALES A. The crystal structure of *N,N*-bis-2-chloroethyl-*N'*-*O*-propylene phosphoric ester diamide monohydrate (endoxan). Acta Cryst B28:2647-2652, 1972.
5. TAKAMIZAWA A, MATSUMOTO S, IWATA T, ET AL. Letter: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985-986, 1973.
6. STRUCK RF, THORPE MC, COBURN WC, JR, ET AL. Cyclophosphamide. Complete inhibition of murine leukemia L1210 in vivo by a Fenton oxidation product. J Am Chem Soc 96:313-315, 1974.
7. VAN DER STEEN J, TIMMER EC, WESTRA JG, ET AL. 4-Hydroperoxidation in the Fenton oxidation of the antitumor agent cyclophosphamide. J Am Chem Soc 95:7535-7536, 1973.
8. TAKAMIZAWA A, MATSUMOTO S, and IWATA T. 4-Hydroxycyclophosphamide anhydro-dimer: revised structure of the Fenton oxidation product of cyclophosphamide. Tetrahedron Lett 6:517-520, 1974.
9. STERNGLANZ H, EINSPAHR HM, and BUGG CE. Letter: Structure and conformation of 4-peroxycyclophosphamide. A cytotoxic oxidation product of cyclophosphamide. J Am Chem Soc 96:4014-4015, 1974.
10. TAKAMIZAWA A, MATSUMOTO S, IWATA T, ET AL. Letter: Synthesis and metabolic behavior of the suggested active species of isophosphamide having cytostatic activity. J Med Chem 17:1237-1239, 1974.

Meeting Participants

ALARCON, R. A.
Cancer Research Institute
New England Deaconess Hospital
185 Pilgrim Road
Boston, Massachusetts 02215

ALLEN, L. M.
Grace Cancer Drug Center
Roswell Park Memorial Institute
Buffalo, New York 14623

ASHBY, R.
Department of Chemistry
Hatfield Polytechnic
Hatfield, Hertfordshire, England

BAKER, M. H.
Institute of Cancer Research
Downs Road
Sutton, Surrey, England

BENCKHUIJSEN, C.
Antoni van Leeuwenhoekhuis
Het Nederlands Kankerinstituut
Sarphatistraat 108
Amsterdam-C, The Netherlands

BERENBAUM, M. C.
Wellcome Laboratories of Experimental Pathology
Variety Club Research Wing
St. Mary's Hospital Medical School
London W2 1PG, England

BONDY, P. K.
Royal Marsden Hospital
Sutton, Surrey, England

BROCK, N.
Pharmacological Department
Asta-Werke A.G. Chemical Factory
Brackwede, West Germany

CAMERMAN, A.
Division of Neurology
University of Washington School of Medicine
Seattle, Washington 98105

CONNORS, T. A.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

COX, P. J.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

CREAVEN, P. J.
Grace Cancer Drug Center
Roswell Park Memorial Institute
Buffalo, New York 14623

DEAN, B.
Ward Blenkinsop Pharmaceuticals Ltd.
P. O. Box 23
Bracknell,
Berkshire RG12 4YS, England

DEWHURST, B. B.
Department of Chemistry
Hatfield Polytechnic
Hatfield, Hertfordshire, England

DONELLI, M. G.
Istituto di Ricerche Farmacologiche "Mario Negri
Via Eritrea 62
20157 Milano, Italy

DRAEGER, U.
Gustav-Embden-Zentrum der Biologischen Chemie
Abteilung für Zellchemie der J. W. Goethe-Universität
Theodor-Stern-Kai 7
6000 Frankfurt am Main, West Germany

FARMER, P. B.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

FOSTER, A. B.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

FRIEDMAN, O. M.
Collaborative Research Inc.
Waltham, Massachusetts 02154

GAMES, D. E.
Department of Chemistry
University College
Cardiff CF1 1XL, Wales

GILBY, E. D.
Institute of Cancer Research
Downs Road
Sutton, Surrey, England

GRIGGS, L. J.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

GRINDEY, G. B.
Department of Experimental Therapeutics
Roswell Park Memorial Institute
Buffalo, New York 14623

HALL, T. C.
Oncology Section
Department of Medicine and the Advanced Therapeutics Program
Los Angeles County-University of Southern California
Medical Center
1720 Zonal Avenue
Los Angeles, California 90033

HAMACHER, H.
Pharmazeutisches Institut der Universität
7400 Tübingen
Morgenstelle, West Germany

HEUSINGER, J.
Organisch-Chemisches Institüt der Universität Mainz
Lehrstuhl 111
Johann-Joachim-Becher Weg 18-20
65 Mainz, West Germany

HILL, D. L.
Kettering-Meyer Laboratory
Southern Research Institute
Birmingham, Alabama 35205

HOHORST, H.-J.
Gustav-Embden-Zentrum der Biologischen Chemie
Abteilung für Zellchemie der J. W. Goethe-Universität
Theodor-Stern-Kai 7
6000 Frankfurt am Main, West Germany

HOUGHTON, P.
Radiopharmacology Department
Royal Marsden Hospital
Sutton, Surrey, England

INCH, T. D.
Chemistry Division
Chemical Defence Establishment
Porton Down
Salisbury, Wiltshire SP4 0JQ, England

JARDINE, I.
Johns Hopkins University School of Medicine
Department of Pharmacology and Experimental Therapeutics

725 North Wolfe Street
Baltimore, Maryland 21205

JARMAN, M.
Institute of Cancer Research
Downs Road
Sutton, Surrey, England

LASTER, W. R.
Kettering-Meyer Laboratory
Southern Research Institute
Birmingham, Alabama 35205

LELIEVELD, P.
Radiobiologische Institut TNO
Lange Kleiweg 151
Rijswijk (Z.H.), The Netherlands

MANDEL, H. G.
George Washington University Medical Center
Department of Pharmacology
2300 Eye Street
Washington, D.C. 20037

MILLAR, J. L.
Institute of Cancer Research
Downs Road
Sutton, Surrey, England

MONTGOMERY, J. A.
Kettering-Meyer Laboratory
Southern Research Institute
Birmingham, Alabama 35205

NORPOTH, K.
Institut für Staublungenforschung und Arbeitsmedizin
Universität Münster/Westphalia
D-44 Münster, Westring 10, Germany

PENN, R.
Department of Chemistry
Hatfield Polytechnic
Hatfield, Hertfordshire, England

PETER, G.
Gustav-Embden-Zentrum der Biologischen Chemie
Abteilung für Zellchemie der J. W. Goethe-Universität
Theodor-Stern-Kai 7
6000 Frankfurt am Main, West Germany

PHILLIPS, B. J.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

PRZYBYLSKI, M.
Organisch-Chemisches Institüt der Universitat Mainz
Lehrstuhl III
65 Mainz, West Germany

ROSS, W. C. J.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

DE RUITER, J.
Radiobiologische Institut TNO
Lange Kleiweg 151
Rijswijk (Z. H.), The Netherlands

RUTMAN, R. J.
Department of Animal Biology
School of Veterinary Medicine
University of Pennsylvania
3800 Spruce Street
Philadelphia, Pennsylvania 19104

SCHEFFLER, G.
Asta-Werke A. G. Chemical Department
Brackwede, West Germany

SCHULTEN, H.-R.
Institut für Physikalische Chemie der Universität Bonn
Wegelerstrasse 12
53 Bonn, Germany

STEEL, G. G.
Institute of Cancer Research
Downs Road
Sutton, Surrey, England

STRUCK, R. F.
Kettering-Meyer Laboratory
Southern Research Institute
Birmingham, Alabama 35205

SYMINGTON, T.
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

SZEKERKE, M.
Elte Szerves Kemiai Intezete
Muzeum körut 4/b
1088 Budapest, Hungary

TAKAMIZAWA, A.
Shionogi Research Laboratory
Shionogi and Company Ltd.
Fukushima-ku
Osaka 553, Japan

TAYLOR, D. M.
Division of Biophysics, Radiopharmacology Department
Royal Marsden Hospital
Sutton, Surrey, England

TEW, K.
Division of Biophysics, Radiopharmacology Department
Royal Marsden Hospital
Sutton, Surrey, England

THOMAS, P
Chester Beatty Research Institute
Fulham Road
London SW3 6JB, England

TISDALE, M. J.
Department of Biochemistry
St. Thomas' Hospital Medical School
Lambeth Palace Road
London SE1 1EH, England

VAN DER STEEN, J.
Antoni van Leeuwenhoekhuis
Het Nederlands Kankerinstituut
Sarphatistraat 108
Amsterdam-C, The Netherlands

VAN PUTTEN, L. M.
Radiobiologische Institut TNO
Lange Kleiweg 151
Rijswijk (Z. H.), The Netherlands

VENDITTI, J. M.
Drug Research and Development, Division of Cancer Treatment
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

VOELCKER, G.
Gustav-Embden-Zentrum der Biologischen Chemie
Abteilung für Zellchemie der J.W. Goethe-Universität
Theodor-Stern-Kai 7
6000 Frankfurt am Main, West Germany

VOIGT, H. C.
Medical Department
Asta-Werke A.G. Chemical Factory
Brackwede, West Germany

WAGNER, T.
Gustav-Embden-Zentrum der Biologischen Chemie
Abteilung für Zellchemie der J.W. Goethe-Universität
Theodor-Stern-Kai 7
6000 Frankfurt am Main, West Germany

WILKOEWITZ, A.
Gustav-Embden-Zentrum der Biologischen Chemie
Abteilung für Zellchemie der J.W. Goethe-Universität
Theodor-Stern-Kai 7
6000 Frankfurt am Main, West Germany
WODINSKY, I.
Arthur D. Little, Inc.
Acorn Park
Cambridge, Massachusetts 02140
YESAIR, D. W.
Biochemistry and Biochemical Pharmacology
Arthur D. Little, Inc.
Acorn Park
Cambridge, Massachusetts 02140

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND 20014

OFFICIAL BUSINESS
PENALTY FOR PRIVATE USE, $300

If your address has changed and you wish to continue receiving this publication, please send your new address and the mailing label from this copy to:

Cancer Treatment Reports
National Cancer Institute
8300 Colesville Rd
Blair Bldg, Rm 3A05
Silver Spring MD 20910

If you do not wish to continue receiving this publication, please notify us at the address noted above and enclose the mailing label from this copy.

A publication of the National Cancer Institute

DHEW Publication (NIH) 76-22